THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Liposomes for mucosal vaccine delivery

Physicochemical characterization and biological application

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Cover illustration: *Dendritic cells (JAWSII cell line) labelled with Hoechst (nuclei) and 10 kDa Dextran-Alexa Fluor 488 (lysosomal compartments). In red, bound to the cell membrane and internalized, are Rhodamine-labelled, CTA1-3Eα-DD-functionalized DOPC-based liposome (referred to as DO-Rho-FP in the thesis).*

Liposomes for mucosal vaccine delivery
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Liposomes are attractive vaccine carriers due to their potential to act as adjuvants, and to the fact that their composition and characteristics are virtually endlessly customizable. However, the precise physicochemical profile of an ideal carrier liposome for mucosal vaccines is still widely unknown, and how different properties affect key steps in the acquisition of protective immunity remains to be elucidated. Additionally, there is no consensus in the field regarding characterization of vaccine formulations, often with incomplete reporting of properties as a result. The focus of this work is therefore twofold: i) to contribute to a better understanding of how the physicochemical profile of vaccine carrier liposomes impacts the development of protective immunity using models at different levels of complexity, and ii) to improve and simplify the physicochemical characterization of liposomes through development and use of new analytical methods. The work in the first area consists of, firstly, an *in vivo* characterization of the biological response to vaccine liposomes carrying a vaccine protein and characterized by varying surface hydrophilicity (PEGylation). This study showed that non-PEGylated vaccine liposomes more efficiently induced local cell- and antibody-mediated immune responses, as well as better protection against a lethal virus challenge than both PEGylated liposomes and free vaccine protein. Secondly, *in vitro* studies focused on how liposome stiffness influences dendritic cells, investigating effects on uptake, antigen presentation and cellular activation. These investigations demonstrated that stiff, gel phase liposomes were able to more efficiently activate dendritic cells and induce significantly higher levels of antigen presentation and co-stimulatory signaling compared to both soft, fluid phase liposomes, and free vaccine protein. The work in the second part comprises two studies: a surface plasmon resonance-based method to characterize the influence on liposome deformation from specific multivalent interactions with supported cell membrane mimics, and a waveguide microscopy technique for characterization of optical properties of individual liposomes. While the latter method might become valuable in the context of quantifying the efficiency of dye labelling of liposomes, the surface plasmon resonance study offered information on how liposome deformation depends on membrane stiffness and ligand-receptor pair density. Taken together, the work presented in this thesis demonstrate the value of multidisciplinary approaches to complex biological and medical challenges.

Keywords: liposomes, nanoparticles, vaccine carriers, influenza, dendritic cells, antigen presentation, cellular uptake, flow cytometry, fluorescence microscopy, surface plasmon resonance

Appended Papers

Paper I

A vaccine combination of lipid nanoparticles and a cholera toxin adjuvant derivative greatly improves lung protection against infection

Valentina Bernasconi, Karin Norling, Sabina Burazerovic, Nagma Parveen, Karin Schön, Anneli Stensson, Marta Bally, Göran Larson, Fredrik Höök, Nils Lycke

In review (Mucosal Immunology)

Paper II

Gel Phase 1,2-Distearoyl-*sn*-glycero-3-phosphocholine-Based Liposomes Are Superior to Fluid Phase Liposomes at Augmenting Both Antigen Presentation on Major Histocompatibility Complex Class II and Costimulatory Molecule Display by Dendritic Cells *in Vitro*

Karin Norling, Valentina Bernasconi, Víctor Agmo Hernández, Nagma Parveen, Katarina Edwards, Nils Y. Lycke, Fredrik Höök, Marta Bally

ACS infectious diseases, 5 (11), 1867-1878, (2019)

Paper III

Vaccine liposome stiffness influences uptake by dendritic cells *in vitro*

Karin Norling, Audrey Gallud, Nils Y. Lycke, Elin Esbjörner Winters, Fredrik Höök, Marta Bally

In manuscript

Paper IV

Characterization of binding-induced liposome deformation using multiparametric surface plasmon resonance

Karin Norling, Nagma Parveen, Marta Bally, Fredrik Höök

In manuscript

Paper V

Effective Refractive Index and Lipid Content of Extracellular Vesicles Revealed Using Optical Waveguide Scattering and Fluorescence Microscopy

Déborah L. M. Rupert, Mokhtar Mapar, Ganesh Vilas Shelke, Karin Norling, Mathias Elmeskog, Jan O. Lötvall, Stephan Block, Marta Bally, Björn Agnarsson, Fredrik Höök

Langmuir, 34 (29), 8522-8531 (2018)

Contribution to the appended papers

Paper I

I performed a large part of the liposome production and characterization and wrote the corresponding parts of the manuscript with input from the other authors.

Paper II

I planned the experimental work together with M.B., performed the majority of it and all the data analysis. I prepared the figures and wrote the manuscript with input from the other authors.

Paper III

I planned and performed the experimental work and the data analysis, prepared the figures and wrote the manuscript with input from the other authors.

Paper IV

I planned the experimental work together with F.H., performed the experiments and the data analysis, prepared the figures and wrote the manuscript with input from the other authors.

Paper V

I contributed to the development of the vesicle labelling protocol used.

Publications not included in the thesis

Mucosal Vaccine Development Based on Liposome Technology
Valentina Bernasconi[*], Karin Norling[*], Marta Bally, Fredrik Höök, Nils Y. Lycke
Journal of Immunology Research, 2016:5482087 (2016)

[*] These authors contributed equally to the work.

List of abbreviations

AFM	atomic force microscopy
APC	antigen presenting cell
CBQCA	3-(4-Carboxybenzoyl)quinoline-2-carboxaldehyde
CCD	charge-coupled device
CLIP	class II-associated invariant chain peptide
CLSM	confocal laser scanning microscopy
Cryo-TEM	cryogenic transmission electron microscopy
CTA1	cholera toxin A1-subunit
CTB	cholera toxin B-subunit
DC	dendritic cell
DD	D-fragment dimer from *Staphylococcus aureus* protein A
DDA	dimethyldioctadecylammonium bromide
DMPG	1,2-dimyristoyl-*sn*-glycerol-3-phosphoglycerol
DMTAP	1,2-dimyristoyl-trimethyl-ammonium propane
DOPC	1,2-dioleoyl-*sn*-glycero-3-phosphocholine
DOTAP	1,2-dioleyl-3-trimethylammonium propane
DPPC	dipalmitoylphosphatidylcholine
DPPS	dipalmitoylphosphatidylserine
DSPC	1,2-distearoyl-*sn*-glycero-3-phosphocholine
ELISA	enzyme-linked immunosorbent assay
EPC	egg phosphatidylcholine
EV	extracellular vesicle
FSC	forward scatter
IL	interleukin
IFN-γ	interferon-γ
LDE	laser Doppler electrophoresis
NTA	nanoparticle tracking analysis
M cell	microfold cell
MHC I	major histocompatibility complex class I
MHC II	major histocompatibility complex class II
MPSPR	multiparametric surface plasmon resonance
M2e	matrix protein 2 ectodomain from influenza A

PA	phosphatidic acid
PAMPs	pathogen-associated molecular patterns
PC	phosphatidylcholine
PBS	phosphate-buffered saline
PE	phosphatidylethanolamine
PEG	poly(ethylene glycol)
PG	phosphatidylglycerol
PI	phosphatidylinositol
PMT	photomultiplyer tube
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
PS	phosphatidylserine
SSC	side scatter
Tc cell	cytotoxic T cell
TEM	transmission electron microscopy
TGF-β	transforming growth factor-β
Th cell	T helper cell
TIRF	total internal reflection fluorescence
TLR	Toll-like receptor
TNF-α	tumor necrosis factor-α

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1
Introduction

The origin of modern vaccinology is often cited as being Edward Jenner's trials with conferring protection against smallpox through controlled infection with the related, though relatively harmless, cowpox virus in the late 18th century.[1] Although inoculation was practiced already long before then, his work marked the beginning of the systematic efforts that lead to the global eradication of smallpox in 1979, in what is arguably one of the biggest achievements of modern medicine.[2] Over these centuries, vaccine technology has developed from the early inoculations, using material from lesions of infected persons; via industrially produced vaccines containing whole pathogens, either attenuated or inactivated, to the use of subunit vaccines containing only well-defined, carefully chosen components of the infectious agent. This development, enabled by the pursuit of understanding the transmission of disease as well as our natural defense against it, the immune system, has led to safer and more effective vaccines. Even so, we are still faced with challenges; the overwhelming majority of diseases have not yet been eradicated, but continue to exist and evolve. Indeed, diseases caused by rapidly mutating pathogens, such as influenza, remain a considerable challenge since vaccine formulations constantly need to be adapted to address the specific strain(s) in circulation at a certain point in time. As a consequence, vaccines cannot be produced far in advance of an actual outbreak, while, at the same time, our modern travel habits make us vulnerable to rapid regional and global spread of infectious diseases.[3, 4] Another factor to consider is that, at the threat of a new pandemic, the go-to means of vaccine distribution, systematic mass-vaccinations through means of injection, poses an additional risk factor for disease transmission, as it gathers large groups of people in limited spaces. Additionally, administration through injection places high demands on vaccine purity, hygiene and access to medically trained personnel. The demands on the next generation of vaccines are thus clear: they should be universal, effective against all strains of a particular pathogen. In addition, administration should be fast and easy, ideally not requiring trained personnel; and, of course, they should be safe and effective.

Universal vaccines can, in principle, be created by making subunit vaccines containing evolutionarily conserved components of the target pathogen.[5] Additional benefits of subunit vaccines are that their composition and production are tightly controllable, usually making them safe, with few manufacturing and regulatory concerns. They are, however, generally less immunogenic than formulations comprising whole pathogens, with the consequence that they must typically be co-administered with immunostimulatory agents, known as adjuvants. Ease of administration could be achieved through the use of mucosal vaccines, which are administered through the mucous membranes, for example, orally or intranasally.[6] Mucosal vaccines have the additional advantage of being characterized by lower demands on purity compared to injected vaccines and can induce a local immune response at the point of entry of the disease-causing pathogen, which systemic administration cannot.[6] However, the mucosal immune system is not easily triggered, since there is a high abundance of potential immunogens, such as pollen or food proteins, to which tolerance is beneficial.[7] Thus, to be effective, current subunit and mucosal vaccine candidates often require large amounts of antigen and strong adjuvants to trigger the immune response.

To improve the performance of mucosal subunit vaccines, the use of particulate carrier systems has been proposed as a promising strategy. The advantage of such systems is that they can improve the bioavailability of antigen, through increased delivery and/or by protecting it from premature degradation, and may additionally have efficient immunostimulatory properties in their own right.[8] Liposomes, spherical lipid bilayer vesicles, have been successfully used as delivery vehicles of various drugs, macromolecules and diagnostic agents in the clinic and are promising candidates as carriers also for vaccines.[9] Liposomes are vastly customizable when it comes to their composition and physicochemical properties, which is one of their main advantages. However, to take full advantage of this flexibility and to best design vaccine vectors capable of inducing an immune response of desired magnitude and type, it is essential to understand how the physicochemical characteristics of liposomes influence their immunogenicity. In order to address such questions, it is important to improve the understanding of the influence that different carrier features have on key events and processes in the development of protective immunity. In the process of gaining such knowledge, which would help rationalize the process of designing future vaccine formulations, it is the hope that one will be able to identify generic vaccine carrier candidates.

The aim of this thesis work has been twofold; firstly, to contribute to a better understanding of the connection between physicochemical properties of liposome-based vaccine formulations and the development of protective immunity. This goal was addressed through studying activation of the immune response as a function of the physicochemical properties of vaccine vectors at different levels of complexity, ranging from *in vivo* investigations of immunogenicity and protection (Paper I) to the use of *in vitro* cell models for investigation of specific key events of interest (Papers II and III). Secondly, the aim was to improve the possibilities to accurately describe the physicochemical properties of liposomes through development and application of surface sensitive techniques for physicochemical characterization of small-scale liposomes (Papers IV and V). Thus, this thesis lays in the intersection of biology, chemistry and physics.

The remainder of the thesis has the following disposition: chapter 2 provides a brief background of the immune system and the challenges and opportunities it provides for vaccination. Chapter 3 introduces the concept of lipids and lipid assemblies and how they can be used as carrier particles, while chapter 4 provides an overview of their various physicochemical properties and the techniques generally available to quantify said properties. Chapter 5 focuses on the use of liposomes as carriers in mucosal vaccines against infectious diseases. Chapter 6 gives more thorough descriptions of the techniques used in the context of this thesis, for characterization of lipid particles as well as of particle uptake and antigen presentation by cells. Chapter 7 summarizes the results obtained during my PhD studies and, finally, chapter 8 presents the future perspectives of this work.

2

Eliciting mucosal immunity through vaccination

The immune system is the collection of defense responses designed for recognition of and protection from "non-self" elements, enabling us to resist attacks from toxins and pathogens, such as viruses, bacteria, fungi and worms, as well as cancer development. The immune system consists of a variety of cells and a multitude of molecules that are either secreted or presented on cell surfaces. The constituents of the immune system can be broadly divided into two cooperative systems that together act to prevent foreign organisms from entering and proliferating in the body: the innate and the adaptive immune systems. Together, these systems provide several layers of defense that interact through a complex interplay of cellular and molecular signaling and interactions.

The first line of defense is the nonspecific, innate immune system. It does not, on its own, provide specific protection against a certain pathogen, but it is a requirement for activation of the adaptive immune response, which provides long-term, specific protective immunity. The innate immune system is composed of cells capable of clearing pathogens and infected cells, as well as of chemical and physical barriers designed to protect us against a foreign invasion. Cell types making up the innate immune system include natural killer cells, which lyse or induce apoptosis in infected and cancer cells. It also includes different phagocytes ("eating cells"), for example: macrophages, neutrophils and dendritic cells, which ingest and degrade viruses, bacteria, foreign particles, dead cells and cell debris, etc. Using pattern recognition receptors, these cells recognize conserved molecular motifs expressed by pathogens. Thus, the innate immune cells do not recognize individual bacteria or viruses, but rather react to common microbial patterns. The innate immune system also hosts important epithelial barriers (e.g. skin and mucous membranes), as well as chemical barriers, such as the enzymes and acidic environment in the stomach. An important contributor to the protective effect of the innate immune response is the complement system, which is a collection of protein and protein fragments that stimulate inflammatory reactions and bind to pathogen surfaces, which triggers cell lysis or promotes phagocytosis. A physical barrier of particular relevance to mucosal vaccination is the mucous membrane, which lines the body cavities and many structures of the body, such

as respiratory and gastrointestinal tracts, including the mouth, nose and lungs. The mucous membranes consist of a layer of connective tissue, known as the lamina propria, overlaid with tightly connected epithelial cells, which is not readily penetrable. On top of this layer of cells there is a layer of mucus, which is a viscoelastic, negatively charged secretion containing mucins and secreted antibodies (immunoglobulins; Igs)[10], as schematically illustrated in Figure 1. The epithelial cells are equipped with cilia: hair-like extensions that move in a coordinated fashion. The movement of the cilia combined with the viscoelastic properties of the mucus creates a directed outward flow of the mucus, trapping and actively transporting foreign matter in what is known as mucociliary clearance.

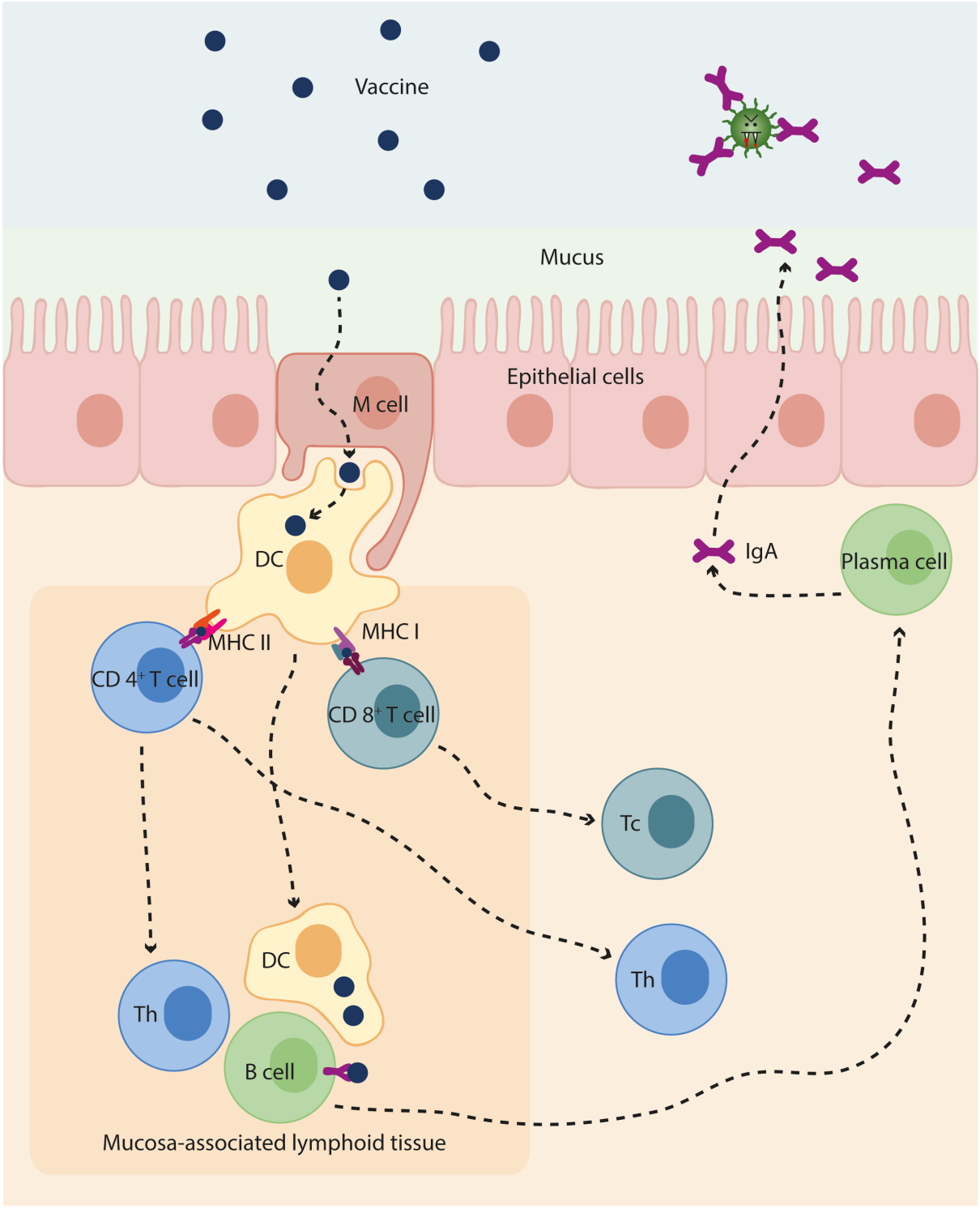

Figure 1. Example of induction of the adaptive immune response through mucosal vaccination. The vaccine needs to pass the mucosal barrier, for example by being taken up by a microfold cell (M cell), which transports it to the other side of the mucous membrane. There, it is available for uptake by dendritic cells (DCs), a highly specialized antigen presenting cell, which take up, process and present antigen on their surface on major histocompatibility complex (MHC) class I and/or II. DCs migrate to nearby mucosa-associated lymphoid tissue where antigen presented on MHC I activates naïve $CD8^+$ T cells, which differentiate into effector and memory cytotoxic T (Tc) cells. Tc cells are specialized in killing damaged cells, such as those infected by viruses or bacteria, or cancer cells. Antigen presented on MHC II activates naïve $CD4^+$ T cells,

which differentiate into effector and memory T helper (Th) cells. Th cells modulate the immune response through cytokine release. DCs additionally traffic antigen to the B cell zone. Naïve B cells are activated by the antigen and co-stimulation from Th cells, and differentiate into memory and effector (plasma) cells. Plasma cells release antibodies, for example secretory IgA, which carries out protective functions, such as binding to surface proteins of pathogens.

The second line of defense, the adaptive, or acquired, immune system is the body's specific response that takes longer to develop, but which, in contrast to the innate immune system, is highly specific to the particular pathogen. Its specificity is the result of unique receptor recognition of foreign molecules, antigens, by T and B cells. More specifically, B cells tend to recognize structural epitopes while T cells recognize linear peptides. The antigen-specificity and formation of memory T and B cells allows the adaptive immune system to retain long-term recognition of the antigen, and in this way facilitate a faster and stronger defense upon subsequent exposures.[11] The purpose of vaccination is to stimulate immunological memory in order to confer long-term protection from infection without giving rise to symptoms of disease.

2.1 Vaccine delivery across the mucosal barrier

Before the adaptive immune system can be exposed to a mucosal vaccine, there is a series of obstacles to overcome. The innate immune system has evolved to repel and degrade foreign matter. Accordingly, mucosal vaccines will, upon administration, immediately encounter the chemical and mechanical cleansing system that is in place at most mucosal surfaces.[7] A first hurdle when developing a vaccination strategy is, therefore, to prevent degradation of the antigen, which is particularly challenging in the case of oral immunization.[12] This, in turn, is the main rationale for improving the resistance of vaccine formulations to degradation, as summarized in section 5.4. A second obstacle for mucosal vaccines is to deliver the antigen to the adaptive immune system, which includes breaching the mucosal barrier.[7, 10] There are three main strategies to increase antigen delivery across the mucosa. The first strategy is to increase mucopenetration, often by using neutrally charged and hydrophilic carrier particles in an attempt to avoid entrapment by the mucus (see section 5.6 for additional details). The second strategy is to increase mucoadhesion, in order to decrease antigen clearance rates. This is often achieved by using particles of a positively charged and hydrophobic nature, as discussed in sections 5.2 and 5.6.[13] The choice between these two opposing strategies depends on the properties of the target mucosa. For example, mucoadhesive carrier particles can be useful when targeting mucosa with a slow mucus turnover rate, while mucopenetrating particles can be used for

traversing thick mucus layers.[14] A third strategy attempts to utilize the body's own system for transport across the mucosal barrier by targeting microfold cells (M cells; see section 5.7). M cells are specialized in phagocytosis and transcytosis, i.e. transport through the interior of the cell, of macromolecules, particles and microorganisms across the follicle-associated epithelium to the lymphoid tissues located in connection to the intestinal and nasal mucosa.[15] For this task, M cells have an intraepithelial pocket, where the antigens taken up from the luminal side are made available to antigen presenting cells (APCs) on the other side of the mucous membrane (Figure 1).[15]

2.2 Antigen presenting cells: linking the innate and adaptive immune systems

Once a vaccine formulation has crossed the barrier of the mucosa, it needs to be recognized by APCs, the bridge between the innate and adaptive immune responses. APCs sample their environment using various uptake mechanisms, including endocytosis, phagocytosis and macropinocytosis.[16] They distinguish self from non-self by recognizing evolutionarily conserved molecular structures exclusively found on pathogens and called pathogen-associated molecular patterns (PAMPs). Examples of PAMPs are bacterial cell-wall components, certain lipids, for example lipid A, and different forms of bacterial and viral nucleic acids.[17-19] APCs use a group of receptors known as pattern recognition receptors for this discrimination process. These receptors include Toll-like receptors (TLRs), C-type lectin receptors and nucleotide-binding oligomerization domain-like receptors.[17-19] Ligand binding to pattern recognition receptors signals danger and starts a signaling cascade, which is critically required for most immune responses. In the absence of sufficient danger signals, the APC will induce tolerance instead of immunity. Pattern recognition receptor agonists are, for these reasons, often used to target vaccine delivery to APCs and as adjuvants to modulate the immune response (see section 5.7).

A type of APC that is exceptionally efficient at taking up antigen, and which is central to the initiation if an adaptive immune response, is the dendritic cell (DC). Once the DC has taken up antigen in the presence of sufficient danger signals, it undergoes maturation, which leads to upregulation of the antigen-processing machinery, followed by antigen presentation. The antigen is processed, degraded into molecular fragments, often short peptides, which are displayed on the cell surface bound to major histocompatibility complex class II (MHC II). MHC II consists of an α- and a β-chain, which are originally assembled together with a chaperone protein known as invariant chain.[20] The invariant

chain stabilizes the MHC II and prevents premature binding of peptide to the complex. Following synthesis, the MHC II is delivered to the plasma membrane, from where it is endocytosed and routed to endosomal-lysosomal antigen-processing compartments by the help of targeting motifs on the invariant chain. In these processing compartments the invariant chain is degraded, leaving behind a small fragment known as class II-associated invariant chain peptide (CLIP) in the peptide-binding groove.[16] Binding of the chaperone protein HLA-DM (H2-M in mice) in the late endosome facilitates removal of CLIP and stabilizes the MHC II, as well as regulates peptide binding, leading to accumulation of MHC II with high-affinity peptides.[21] Finally, the peptide-MHC II complex is presented on the cell surface. However, the complex does not necessarily remain there; on the contrary, in immature DCs there is a rapid turnover of peptide-MHC II complexes as they are continuously marked for internalization by addition of the regulatory protein ubiquitin (ubiquitination) by the enzyme MARCH1.[22, 23] Upon DC maturation, MARCH1 expression is downregulated, leading to an increase in the stability of presented peptides.[22-26] Furthermore, the endocytic activity is decreased in mature DCs, especially of non-specific uptake mechanisms, such as macropinocytosis and receptor-independent phagocytosis, which involve turnover of large amounts of membrane.[16, 27, 28] Together, this leads to a redistribution of peptide-MHC II complexes from endosomal-lysosomal compartments in the immature DC to the plasma membrane, where they are comparatively stably presented on the mature DC.[16] Antigen presentation on major histocompatibility complex class I (MHC I) molecules is, in most cell types, reserved for endogenous peptides and used to signal normal cellular function. However, DCs have the ability to present also extracellular antigens on MHC I in a process known as cross-presentation. The importance of this process in development of cell-mediated immunity is discussed further in section 2.3. Activated, mature DCs migrate to the draining lymph node where T and B cells reside in separate zones and the presented antigen is recognized by T cells. Initiation of a primary T cell response via activation of naïve antigen-specific T cells, referred to as priming of the T cells, can only be done by dendritic cells (DCs). For this reason, DCs are critically important for vaccination and a target of choice for vaccine delivery (see section 5.7).[29] There are three main DC subtypes: conventional DC1, conventional DC2 and plasmacytoid DC, which represent distinct functional lineages.[30-32] The conventional DC subsets are highly efficient at antigen processing; the conventional DC1 subset has a high capacity to cross-present antigen on MHC I, while the conventional DC2 subset presents antigen on MHC II. The

plasmacytoid DC subset is instead specialized at sensing and responding to viral infection by rapidly producing large quantities of cytokines.[31] The DC response to changes in vaccine vector properties was the main focus of Paper II and III, specifically effects on uptake (Paper III) and antigen presentation, MHC II loading, and co-stimulation (Paper II).

2.3 Inducing and tuning cell-mediated immunity

As shown in Figure 1 and further detailed in section 2.2, DCs play a key role in the primary immune response, as they activate naïve T cells in the draining lymph node. The activated T cells then undergo strong expansion and subsequently differentiate into different subtypes as effector or memory T cells, which are essential for long-term immunity. A key event required for T cell priming is the binding of a T cell receptor to the foreign peptide in complex with MHC I or II molecules on the DC surface. In addition to this, T cells require surface-bound co-stimulatory molecules, such as CD80 and CD86, as well as released cytokines, all expressed by the activated DC. Together, these activation signals act in concert to initiate and tune a specific T cell response. Firstly, recognition of peptide-MHC II complexes leads to the activation of $CD4^+$ T cells into T helper (Th) cells, whose main function is to organize the immune response and support B cell and $CD8^+$ T cell immunity by releasing cytokines. Cross-presentation of antigens as peptide-MHC I complexes leads to priming of naïve $CD8^+$ T cells, which can develop into cytotoxic T (Tc) cells that are important for the killing of virus-infected cells (see Figure 1).[11] Particulate delivery systems have been shown to increase cross-presentation efficiency, making carrier particles useful for vaccines for Tc cell induction.[33-35] Secondly, cytokines released by DCs strongly influence the type of T cells that are generated. Th cells are divided into functional subsets that are all generated from the naïve $CD4^+$ T cell population. Different cytokines trigger polarization of the Th cells into subsets characterized by the different cytokines they produce and thus, which type of immune response they promote (see Table 1). Most notable among these subsets are the Th1, promoting cell-mediated immunity, and the Th2, promoting antibody-mediated, also known as humoral, immunity.

Table 1. An overview some of the most commonly described T helper (Th) subsets, of relevance for the topic.[36, 37]

Th subset	Triggering cytokines	Cytokines released by subset	Subset role
Th1	IL-12, IFN-γ	IFN-γ, TNF-α	To activate a cell-mediated immune response against intracellular pathogens (e.g. viruses and intracellular bacteria).
Th2	IL-4	IL-4, IL-5, IL-13	To activate a humoral immune response against extracellular pathogens (e.g. extracellular bacteria and parasites).
Th17	TGF-β, IL-6, IL-23	IL-17, IL-21. IL-22, IL-23	To help maintain mucosal barriers and clear pathogens at mucosal surfaces.
Treg	IL-10	IL-10, TGF-β, IL-35	To modulate and suppress the immune response to prevent excessive and harmful immune- and inflammatory responses.
Tfh	IL-21, IL-6	IL-21	To promote B cell differentiation and aid in selection of high-affinity B cells to promote strong, long-lived humoral immunity.

2.4 Inducing humoral immunity

Humoral, antibody-mediated, immunity is generated by B cells that develop into plasma cells that secrete the actual antibodies, which are large proteins able to bind to specific epitopes on antigens. Important effector functions of antibodies include neutralization of toxins and microbes, activation of the complement system and opsonization of target cells, a process by which a pathogen is marked for destruction by phagocytes.

Antibody-antigen recognition is highly specific due to the fact that the chemical properties of the amino acid sequence forming the antigen-binding site of the antibody geometrically and physicochemically match regions on the corresponding epitope on the antigen. This allows formation of a multitude of electrostatic and hydrophobic interactions and hydrogen bonds, the sum of which is a high-affinity interaction between an antibody and its antigen.[11] Antibodies are classified into five different immunoglobulin classes, or

isotypes, according to their heavy chain, and each class is adapted to different functions in different compartments of the body (Table 2). Of special relevance in the context of vaccination are IgG, the main serum antibody; and secretory IgA, which is produced by plasma cells in the lamina propria in the mucous membrane.[11] It has been found that secretory IgA is effectively stimulated only by local mucosal immunization, highlighting the advantages of mucosal vaccination.

Table 2. An overview of the different mammalian antibody classes, or isotypes.[11]

Class	*Role*
IgG	The most abundant class found in blood and extracellular fluid. Efficient opsonin and activator of the complement system.
IgA	Mainly found in secretions and at epithelial surfaces. It is the main antibody secreted by mucosal lymphoid tissues and its main function is as a neutralizing antibody.
IgM	The first antibody to be produced in an immune response; generally, has low affinity, but as it is secreted in pentamers it has high avidity. Secreted, it is found in blood, but generally not in tissues due to its large size. Efficient complement activator.
IgE	Present only in low levels in blood and extracellular fluid. Binds to receptors on mast cells, which induces reactions meant to expel infectious agents; such as coughing, sneezing and vomiting. Involved in defense against infections by multicellular parasites and in allergic reactions.
IgD	Expressed on the surface of mature B cells but its function is so far unknown.

Antibodies are produced by B cells (see Figure 1) and exist in two forms: a free, secreted form and a cell-membrane bound form known as the B cell receptor. The B cell receptor is crucial for the activation and differentiation of naïve B cells into plasma cells and memory B cells, which maintain long-term protection. B cells are activated by recognition of antigen with the B cell receptor in the B cell zone in the lymph node; this leads to internalization of the antigen, which after degradation can be presented as peptide complexed with MHC class II molecules on the surface of the cell. Hence, also B cells can act as APCs. But, more importantly, the activated B cell undergoes strong expansion in the lymph node and this requires interactions with activated follicular helper T (Tfh) cells. B cell responses that are independent of this T cell help are infrequent and require bacterial antigens with repetitive

epitopes: certain polysaccharides, lipopolysaccharides and polymeric proteins. However, these responses often give rise to low affinity antibodies and no memory development.[11] In contrast, during T cell-dependent B cell responses, the immunoglobulin undergoes affinity maturation; mutations are introduced in the variable regions (somatic hypermutation) and affinity-based selection by Tfh cells. Thus, B cells producing higher-affinity antibodies are positively selected in the germinal center of the B cell zone.[11] Furthermore, B cells undergo class switching, a process that changes the isotype of the antibody produced. During this process, the antibody retains its antigen-specificity while its constant heavy chain is changed. Since the different classes host different functions, with IgA antibodies particularly effective at mucous membranes, while IgG antibodies are preferentially found in serum, a vaccine can be designed or given by a certain route to stimulate more of IgA than IgG, or vice versa.

2.5 Assessing the immune response

Historically, vaccine development has most often focused on antibody-mediated immunity.[38] Indeed, antibodies are crucial for preventing infection since they can block the surface proteins of pathogens and thus prevent them from binding to, and entering, host cells. Cell-mediated immunity, on the other hand, is necessary for attenuation and clearance of intracellular infections and critically required for most B cell responses. Therefore, the aim of vaccines is to achieve both humoral and cell-mediated immune responses, both locally and systemically.[38] The intensity and quality of an induced immune response is typically assessed by measuring a set of biological markers. Different types of antibodies and cytokines, such as interleukins (ILs), are commonly quantified and indicate activity of certain cells of the immune response. Some of the more commonly used markers and what they indicate in the context of vaccine development are summarized in Table 3. In Paper I, a thorough *in vivo* investigation of protection and immunogenicity was performed, which included quantification of both antibodies and cytokines. In Paper II, an *in vitro* DC model was used and release of certain relevant cytokines was quantified.

Table 3. Commonly used markers of immune activation following immunization, as well as ones of particular relevance for the work presented in this thesis.[11, 36, 39-41]

Marker	*Type of molecule*	*Role*
IgG	Antibody	Indicates activation of systemic humoral immunity.
IgA	Antibody	Indicates activation of mucosal/local humoral immunity.
IFN-γ	Cytokine	Characteristic of a Th1 response, promoting cell-mediated immunity. IFN-γ is an important activator of APCs, especially macrophages, and inducer of MHC molecule expression and components of the antigen processing machinery. Induces antibody class switching and suppresses the Th2 response.
IL-1β	Cytokine	Activates T cells, B cells and macrophages. Induces fever.
IL-2	Cytokine	Drives T cell proliferation and differentiation into memory and effector T cells. IL-2 is crucial for the survival of activated T cells and has a central role in the initiation of an adaptive immune response.
IL-4	Cytokine	Characteristic of a Th2 response, promoting humoral immunity. Activates B cells and induces $CD4^+$ cell differentiation into Th2 cells. Induces antibody class switching to IgE.
IL-6	Cytokine	Activates T and B cells, drives their proliferation and differentiation, stimulates antibody production.
IL-10	Cytokine	Regulatory cytokine, suppresses macrophage functions and inhibits proliferation of, and cytokine production by, $CD4^+$ T cells.
IL-17	Cytokine	Characteristic of a Th17 response, important for maintaining mucosal barriers and clearance. IL-17 has an important role in proinflammatory responses and induces the production of many other cytokines.
IL-22	Cytokine	Plays an important role in maintenance and defense of epithelial barriers.
IL-23	Cytokine	Induces proliferation of Th17 memory cells and increases IFN-γ production.
IL-27	Cytokine	Drives regulatory T cells of the Tr1 subtype and has an inhibitory effect on Th1, Th2 and Th17 subset functions, mediated by IL-10 production.

3
Lipids and lipid self-assemblies

Lipids are an important building block of many living organisms in that they are the main constituents of the cellular membranes. The membranes are thin, on the order of 5 nm thick, fluid films to which lipids provide a structure that allows for lateral movement of incorporated proteins and other biomolecules.[42] The membranes form selectively permeable barriers that maintain appropriate intracellular concentrations of a vast number of molecules and ions, thus delineating individual organelles as well as the entire cell from the outside.[42] Among the various lipids found in living organisms, phospholipids are the ones most abundantly found in cell membranes. This type of lipid is also the main constituent of many man-made lipid structures, including vaccine vectors.[43, 44] They are amphiphilic, made up of a hydrophobic tail consisting of two fatty acids linked by a glycerol backbone to a hydrophilic headgroup made up of phosphate and potentially another organic molecule (Figure 2A). Based on their headgroup, naturally occurring phospholipids can be sorted into 6 categories: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG) or phosphatidic acid (PA). PS, PI, PG and PA are negatively charged while PC and PE are neutral but zwitterionic. The possibility to chemically modify both the head group and the tail region gives the possibility to synthesize phospholipids tailored to specific requirements. In this way, positively charged lipids have been created, for example 1,2-dioleyl-3-trimethylammonium propane (DOTAP), 1,2-dimyristoyl-trimethyl-ammonium propane (DMTAP) and dimethyldioctadecylammonium bromide (DDA).

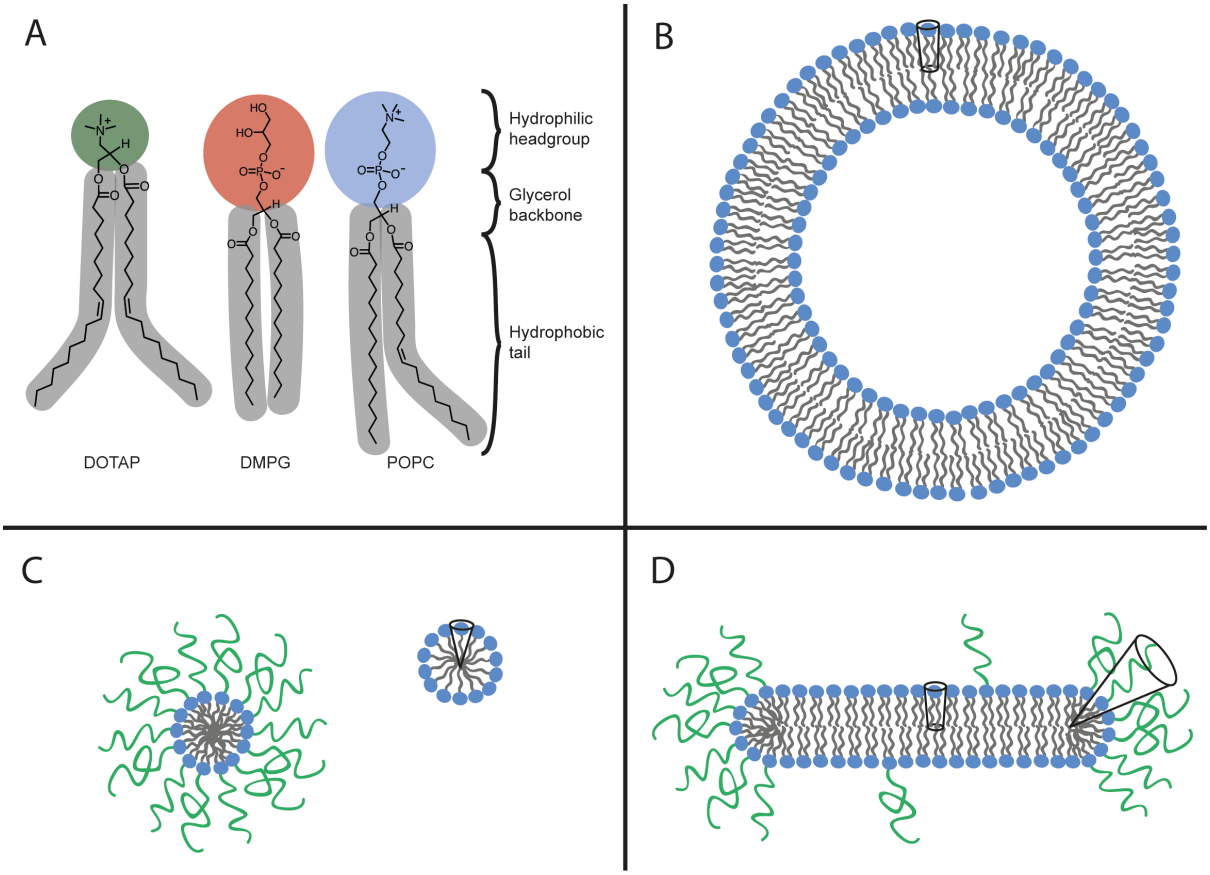

Figure 2. (A) Examples of lipids with different properties: 1,2-dioleyl-3-trimethylammonium propane (DOTAP), with a positively charged headgroup and one unsaturation in each alkyl chain, 1,2-dimyristoyl-*sn*-glycerol-3-phosphoglycerol (DMPG), with a negatively charged headgroup and saturated alkyl chains, and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), with a zwitterionic headgroup and one unsaturated and one saturated alkyl chain. (B) A liposome is a spherical bilayer structure consisting of lipids with a cylindrical geometry. (C) Spherical or elongated micellar structures consists of lipids with a conical geometry. (D) Lipodisks, bilayer segments with edges stabilized by micelle-forming lipids, can be formed by mixing bilayer- and micelle-forming lipids at certain ratios.

In an aqueous environment, amphiphilic molecules such as phospholipids self-assemble into different types of molecular assemblies where the hydrophobic parts face each other and form a protected compartment with the hydrophilic parts facing the aqueous solvent.[45] Such self-assembled amphiphilic structures exist in an equilibrium state; they are often not definite and constant but rather soft and fluid, with the constituent molecules exhibiting a high degree of movement within the aggregate and, under certain conditions, also between individual structures.[46-49] The size and shape of such structures are therefore often not sharply defined, but rather distributions that can be fairly broad.[46] The most energetically favorable, and therefore often most likely, structures adopted by amphiphiles depend on concentration and physicochemical properties of the amphiphiles

as well as on environmental factors such as the temperature, the ionic strength and the pressure, although the effect of the latter is generally small.[45] Among the properties of amphiphilic molecules the geometry is of particular interest as it affects the molecular packing and thus the type of structures, or mesophases, that are formed. There are essentially two basic building blocks, micelles and bilayers, that make up the different self-assembled structures that can be formed. Micelles are spherical or cylindrical structures with a hydrocarbon core and a surface consisting of hydrophilic groups (Figure 2C). Amphiphiles with a conical geometry i.e. with large headgroup area, often due to a charged or bulky headgroup, and a comparatively small hydrophobic tail tend to form micellar structures. Amphiphiles with a tail region that is large compared to the headgroup, for example when the hydrophobic tail consist of two alkyl chains, have a more cylindrical geometry, and will tend to form bilayer structures (Figure 2B).[46] So called lipid bilayers consist of two layers of amphiphiles assembled with the hydrocarbon chains facing each other, thereby protected from thermodynamically unfavorable interactions with the aqueous solution by the hydrophilic headgroups. Self-assemblies containing micellar or bilayer structures, or both, can form large, interconnected isotropic structures or liquid crystalline phases, or remain as discrete entities, thereby forming particles. Such particles are of interest for many types of drug delivery applications and for vaccine delivery purposes.[50]

One type of lipid-based particle commonly encountered is the vesicle, also called liposome. In this case, the energetically unfavorable outer edges of planar bilayer sheets are eliminated by forming a hollow sphere. The sphere consists of either a single or multiple phospholipid bilayers and liposomes are accordingly classified as unilamellar; multilamellar, if the bilayers are concentric; or multivesicular, if they are not. Liposomes where discovered in 1965 by Bangham et al. and in the 1970's they were for the first time explored for drug delivery purposes and as immunological adjuvants.[51-53] Liposomes have since been extensively explored as vaccine vectors, with several examples being in commercial use and clinical trials.[12, 54, 55] Particles which contain elements of both micelles and bilayers may form if conical and cylindrical lipids are mixed. One such example of direct relevance to this thesis, is the lipodisk, which was first described by Edwards et al. in 1997.[56] Lipodisks are flat, single bilayer disks, comprising bilayer-forming lipids with micelle-forming lipids eliminating the energetically unfavorable contribution from the high-curvature edges (Figure 2D). Lipodisks have been used as

membrane mimics and for drug delivery purposes but until now (in Paper II) they have, to the best of our knowledge, not been considered for use as vaccine vectors.[57-59]

3.1 Functionalization and labelling of lipid particles

The choice of lipid composition governs the type of particles formed, as well as their physicochemical properties. In addition to this, particles may be further customized through the introduction of different functional molecules. Association of such molecules may be done using a variety of different strategies. Hydrophobic molecules may be incorporated in the hydrophobic region of the particle, while hydrophilic molecules can be either encapsulated in the aqueous core of liposomes, or associated to the particle surface. Surface association can take place by covalent attachment or spontaneous association of the antigen to the surface through adsorption or electrostatic interaction or, alternatively, the functional molecule can be attached to a hydrophobic anchor that inserts into the particle. Having these different alternatives is especially advantageous when using lipid particles as vaccine carriers, as these different options allow association of a variety of antigens with different properties. Apart from antigen association, lipid particles may be functionalized with, for example, cell-targeting elements or bioadhesive polymers (chapter 5.6 and 5.7), as well as various types of labels to enable visualization of particles or particle components beyond the boundary imposed by the diffraction limit. This type of visualization is commonly achieved by the introduction of fluorescent labels. The concept of fluorescence is explained in chapter 6.1, while here, the strategies available to introduce fluorescent labels (the generalities of which are applicable also to many other types of functional molecules) into lipid particles are explained and discussed. Firstly, one might introduce the label already when producing the particle, or alternatively, the label can be introduced into already finished particles. Secondly, there is a variety of different kinds of labels, which targets various structures or molecules, such as lipid membranes or proteins associated with the particle.

Lipid membranes are often labelled during production by including a small percentage of lipids that carry a fluorescent molecule, either attached to the headgroup or to the tail region; alternatively, water-soluble fluorescent molecules may be encapsulated in the aqueous core of liposomes. Another strategy is to label the lipid particles post-production using a membrane-inserting dye, such as PKH; dialkylcarbocyanines, such as DiO, DiD and DiI; or octadecyl rhodamine B chloride. These are dyes with a lipophilic group which,

as the name suggests, inserts itself in lipid membranes. The precise nature of the lipophilic group affects how easily the dye inserts itself in the membrane and how much it tends to leak out or transfer. This type of dye is generally designed as a means to label cellular membranes, but can, with some modification to the labelling protocol, be used for lipid particles. An advantage of the lipophilic dyes is that one does not need to specially produce labelled particles, but can simply add the dye to existing ones. This is a great advantage for work with biological particles, such as extracellular vesicles (EVs) and viruses.[60-66] Additionally, one may claim some control over the directionality of the dye, as it inserts from the outside of the particle, while, if one forms liposomes with fluorescent lipids, approximately half of the fluorophores will be directed towards their interior, assuming the same lipid composition of the inner and outer parts of the bilayer. Disadvantages of the membrane-inserting dyes include the need for separation of excess dye, which tends to lead to some particle loss. It has also lately been revealed (by Lubart et al., in review) that the dye insertion efficiency may depend upon the properties of the membrane into which it is inserting, something that calls for careful characterization of the labelling if one wishes to compare different types of particles.[67]

As an alternative, or complement, to labeling of the lipid membrane, any proteins associated with the lipid particles may also be fluorescently labelled. Proteins can be recombinantly produced and fused with fluorescent moieties, such as green or red fluorescent protein. Alternatively, post-production labelling of proteins can be done by covalently attaching fluorescent tags using a variety of different chemistries, or by immunolabelling certain epitopes with fluorescently labelled antibodies.

4
Physicochemical characterization of liposomes

Considering the large variety in lipids as well as the multitude of ways in which they may assemble, lipid particles can exhibit a wide range and combination of physicochemical properties. This chapter is intended to give an overview of the various physicochemical properties commonly (or sometimes not so commonly) reported for lipid particles in vaccine delivery contexts, and the techniques available to assess them. A more thorough description of the techniques used in this thesis is found in chapter 6.

4.1 Size and morphology

At first glance, size is perhaps the most intuitive of the physicochemical properties described here. However, there is a variety of meanings of the concept of size, dependent on the context and the underlying principle of the technique used for the size determination of the particle.

Most nanoparticles are too small to be resolved using conventional light microscopy. They can however be still be detected and their size can be determined by other types of optical techniques. Dynamic light scattering is perhaps the most common particle sizing technique, which, similarly to the more recent nanoparticle tracking analysis (NTA) technique, relies on optically probing the Brownian motion of particles in suspension.[68-70] Both techniques thus relate size in terms of hydrodynamic radius or diameter. The hydrodynamic radius, or Stokes radius, of a particle is not its actual physical size but the radius of a hard sphere with the same diffusion rate. Dynamic light scattering requires relatively large sample volumes and tends to provide results biased towards large particles, while NTA, being a single particle technique, is better at resolving polydispersity, but generally provides worse statistics.[68]

Another sizing technique, which, similar to NTA, provides single particle information is tunable resistive pulse sensing. This technique is based on the Coulter principle, with particles in an electrolyte-containing suspension passing through a conductive pore.[71] The transient increase in impedance as electrolytes are displaced by the particle in the pore

causes a measurable drop in the current across the aperture, corresponding to the particle volume. A disadvantage of this method compared to direct methods, such as dynamic light scattering and NTA, is the need for calibration using particles of a known size. This means that the quality of the size determination is dependent on the particles used for calibration and, in extension, the method originally used to characterize them.

A technique that, in addition to size, offers morphological information, for example about particle shape and structure, is electron microscopy.[72] Electron microscopy can be divided into scanning and transmission electron microscopy (TEM), both providing down to sub-nanometer resolution. In scanning electron microscopy, the electron beam is, as the name implies, scanned across the specimen and the emitted secondary electrons provide information about surface topography. Generally speaking, the technique demands a conductive sample, often requiring specimens to be coated with a thin layer of metal. In TEM however, it is instead the electrons transmitted through the specimen that provides information about the atomic number and thickness of the sample and can be used to probe internal structure, such as liposome lamellarity. Such measurements require specimens to be thin enough to be electron transparent and generally take place in vacuum, which means that samples in aqueous solution must be frozen (see section 6.3 for further details). Thus, both types of electron microscopy places high demands on sample preparation, which might in turn introduce artefacts.[73] Moreover, TEM resolves only structures with high mass and/or thickness, which, in this context, generally means that only the lipid structures are visualized, while any associated proteins or polymers, such as PEG, are very difficult to visualize.

Another technique that can be used to obtain morphological information in addition to size is atomic force microscopy (AFM).[74] The technique utilizes a very fine tip attached to a cantilever. As the tip is brought into very close proximity with a surface-bound sample, the cantilever is deflected; the magnitude of the deflection and/or the force with which it interacts as it approaches is recorded, depending on the mode of operation.[75] The tip is scanned over the specimen in order to image its topography. AFM is, similarly to NTA and electron microscopy, a single particle technique, and has a resolution in the z-direction approximately comparable to that of electron microscopy. An advantage over electron microscopy, however, is that AFM can be performed on samples in the liquid state, allowing for studies of dynamics.

The structure and dynamics of molecules in liposomes can be examined using nuclear magnetic resonance spectroscopy. With this technique the phenomenon of nuclear spin, the magnetic moments of atomic nuclei, is utilized by applying a magnetic field and observing the frequency of the resonant electromagnetic field of the studied molecules.[76] ^{31}P nuclear magnetic resonance spectroscopy takes advantage of the presence of phosphorus in the phospholipids that generally make up liposomes (see chapter 3) and is especially useful as it allows for determination of liposome lamellarity.[77] To do this, the signal from the exterior phosphate groups are quenched using Mn^{2+}, and the lamellarity is determined using the ratio of the signal before and after quenching. In general, nuclear magnetic resonance spectroscopy is non-invasive, highly sensitive and offers high resolution. However, the Mn^{2+} quenching-method for lamellarity determination has proven sensitive to the types of liposomes as well as the concentrations of Mn^{2+} and the buffer.[77]

Finally, small-angle X-ray scattering detects scattering at low angles upon sample illumination with a monochromatic X-ray beam. The technique can be used to provide information about nanometer-scale structure, organization and thickness of lipid bilayers, as well as information on vesicle size.[78-80] A drawback of the technique is that it, in analogy to electron microscopy, AFM and nuclear magnetic resonance, requires specialized equipment and, especially, highly trained personnel.

Worth noting is that single particle techniques such as NTA, tunable resistive pulse sensing, electron microscopy and AFM have the power to provide more information than ensemble average techniques. When studying single particles, one has the opportunity to observe and resolve different subpopulations of the property of interest. Indeed, recent developments in new forms of single particle detection demonstrate efforts at moving away from ensemble average techniques.[81-83]

4.2 Zeta potential

The surface charge of particles is generally assessed in terms of zeta potential, a measure of the electrostatic potential difference between the bulk fluid and the slipping plane at what is called the diffuse electric double layer that surrounds the particle (Figure 3). The double-layer consists of stationary (in relation to the particle) fluid and differently charged

ions distributed in the near vicinity of the particle surface, thus shielding the surface charge. The thickness of the double layer is also known as the Debye length and is defined as the distance from the surface where the potential has fallen to 1/e of its value at the surface.[45] The magnitude of the zeta potential depends on the surface charge of the particle and the concentration of ions within the double layer, which in turn depends on other factors, such as the ionic strength and pH of the dispersion medium.

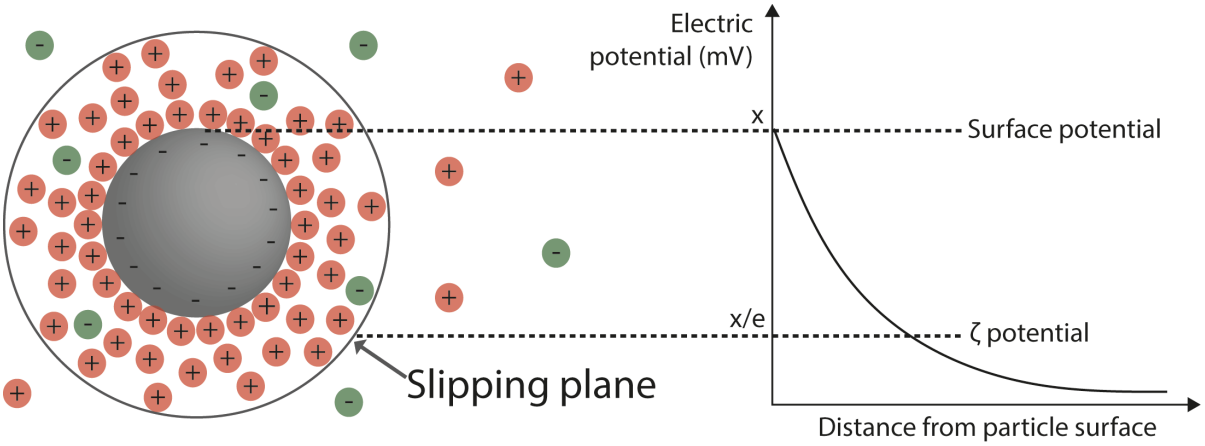

Figure 3. The zeta potential is difference in electric potential between the bulk fluid and the slipping plane at the limit of the diffuse electric double layer of loosely associated fluid and ions surrounding a charged particle. The slipping plane is located where the electric potential has dropped to 1/e of its value at the particle surface.

The zeta potential is commonly assessed by electrokinetic measurements, in which motion of charged particles is induced by the application of an electric field. The speed of this motion is directly proportional to the zeta potential of the particles and can be assessed in conjunction with size determination using optical techniques reliant on particle mobility, namely dynamic light scattering and NTA (see section 4.1). Furthermore, tunable resistive pulse sensing (see section 4.1) can also be used for zeta potential measurements by using an electric field to induce particle passage through the pore. Since particle velocity in an electric field relates to the zeta potential, the rate of particle passage or, to gain single particle information, the width of the current drop caused by an individual particle passing through the aperture, corresponding to the particle's speed, is recorded.[71, 84]

4.3 Antigen content

For lipid particles in the context of vaccine delivery, determination of the antigen load or content is often of interest. Broadly speaking, protein determination can be divided into two types, unspecific and specific protein quantification.

In unspecific, or total, protein quantification, no discrimination between different proteins in the sample is made. There is a variety of assays available for this type of protein detection: perhaps most notable are the Lowry,[85] the bicinchoninic acid[86] (often referred to as BCA) and the Bradford[87] assays, which all rely on chemical reactions causing a colour change. The change in colour is accompanied by a change in absorbance at a certain wavelength, which is proportional to the protein concentration. Worth to note is that, due to the differing nature of the reactions involved, the assays have varying buffer compatibilities. For example, the Bradford assay is sensitive to the presence of detergents, making it unsuitable in cases where proteins are incorporated in the lipid bilayer or encapsulated in the interior of lipid particles, which then need to be disrupted to release the proteins. Simultaneously, the Lowry and bicinchoninic acid assays are sensitive to the presence of chelators, such as EDTA, due to the involvement of copper ions in the detection reactions. A more recent protein assay, which is not sensitive to these particular additives, is the fluorometric 3-(4-Carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) assay, which is presented in more detail in chapter 6.1.[88] Additionally, in some cases it is possible to detect the absorbance at 280 nm of tryptophan, tyrosine and/or disulfide-bonded cysteine residues using UV-vis spectrophotometry, and calculating the protein concentration using either a standard curve or a known absorptivity value for a particular protein.[89]

Techniques for specific protein detection often rely on immunolabelling, using antibodies that specifically interact with the particular protein of interest. The most commonly used techniques for this purpose are enzyme-linked immunosorbent assay (ELISA) and Western blot.[42] ELISA utilizes a detection antibody with a conjugated enzyme that converts an added substrate to a coloured product, which can then be optically detected. The enzyme can perform this conversion many times, allowing it to act as a signal amplifier. In Western blotting, proteins in a complex mixture are first denatured and separated based on molecular weight (and potentially also their isoelectric point) using gel electrophoresis. They are then transferred (blotted) to a membrane, where they are immunolabelled with one out of several detectable probes. Of course, these approaches require access to relevant detection antibodies, which are not available for all proteins. In the case where antibodies are not available, mass spectrometry is an attractive alternative.[42] In mass spectrometry, proteins and protein fragments are ionized and identified using their mass-to-charge ratio

with the help of comprehensive databases. The technique is very powerful for protein identification, but it is also increasingly used for quantification. It does, however, require specialized equipment and knowledge.

4.4 Particle stiffness

The deformability of lipid membranes, in terms of bending rigidity (an intrinsic property; not dependent on geometry), is most commonly measured on large liposomes (~10 μm in diameter) using either fluctuation spectroscopy, in which thermal fluctuations of lipid membranes are observed using optical microscopy, or through means of mechanical manipulation: often by micropipette aspiration, electrodeformation or through the use of optical tweezers.[90] Additionally, it is possible to measure bending rigidity using a number of scattering techniques, including diffuse X-ray scattering, neutron and X-ray reflectivity as well as neutron spin echo combined with dynamic light scattering.[90] However, in the case of small surface-adsorbed liposomes (~100-200 nm diameter) the internal pressure, in addition to the membrane rigidity, greatly influences the effective stiffness.[91] For such small liposomes the abovementioned techniques are unsuitable. Therefore, AFM indentation has become a popular tool to study the mechanical properties of small vesicles.[91-94] Furthermore, AFM used in imaging mode has been used to quantify surface-induced deformation of liposomes.[95] However, AFM on small liposomes is highly demanding in terms of skill and time. Each liposome needs to be individually probed, making it a work- and time-intensive technique, and limiting the possibilities to obtain good statistics. Thus, an interesting recent development is the use of multiparametric surface plasmon resonance (SPR) for the purpose of studying liposome-surface interactions, an approach which will be expanded upon within this thesis (in Paper IV).[96] Multiparametric SPR is, as opposed to AFM, an ensemble average technique and is therefore less suitable to resolve variety within samples. It does, however, have the considerable advantage of being fairly rapid, both in terms of measurement and analysis.

5
Liposomes for mucosal vaccine delivery

Liposomes have attracted considerable interest as carriers for mucosal vaccine delivery for a number of reasons. First of all, the membrane composition is easily adjustable and membrane constituents can be synthetic or sourced from vastly different organisms, which invites to biomimicry. Thus, attempts have been made to enhance the immunogenicity of a lipid formulation by choosing membrane components with archaeal, bacterial or viral origins.[97-103] Conversely, by choosing endogenous lipids, vectors can be made entirely innocuous: biodegradable, non-toxic and non-immunogenic.[44, 104] Oftentimes, highly immunogenic formulations are also toxic, and creating a formulation that is effective without unwanted side effects is a challenge. One may therefore attempt to systematically address which particular physicochemical properties are at the root of the immunogenic effect to be able to create formulations with the desired properties but without toxic constituents.

An advantage of lipid-based vaccine formulations is that the physicochemical properties of the lipid particles are vastly adjustable. The membrane properties can be tuned by altering the lipid composition: the surface charge of the particles is largely affected by the characteristics of the lipid headgroup, and the degree of hydrophilicity can be tuned by addition of polymers such as poly(ethylene glycol) (PEG).[9, 105] The headgroup charge, along with the length and degree of saturation of the alkyl chains of the tails, further influences the transition temperature of the lipids, which in turn determines whether a lipid membrane exists in a gel or fluid phase state at a certain temperature. Moreover, if a membrane consists of a mixture of lipids, phase separations can occur, resulting in heterogeneous distribution of different lipids. The stability of a membrane, i.e. its resistance to degradation, is affected by its fluidity and permeability as well as its bending rigidity, which are in turn influenced by the same lipid characteristics. Incorporation of cholesterol is a common way to modulate the membrane permeability, fluidity and rigidity, which influence the liquid-to-gel phase transition temperature and stability of the formulation.[50, 106] Liposome size and morphology/lamellarity can be tailored by altering the

manufacturing method; for lipodisks, the size is affected by both method and composition.[106, 107]

An inherent property of lipid particles, which makes them useful as antigen carriers, is that they contain both hydrophobic and hydrophilic regions, allowing for a variety of coupling strategies. Hydrophobic peptides or proteins can be incorporated into the hydrocarbon center of bilayers or micelles, while hydrophilic molecules can be coupled to the surface of lipid particles or encapsulated in the aqueous core of liposomes (see Figure 4C). In cases where the lipid particles are included in a vaccine formulation solely as adjuvants, they may simply be co-administered with the antigen.

With the possibility to tailor both preparation method and composition, as well as ways of incorporating antigen in the formulation, there are virtually endless possibilities in the production of lipid structures with varying properties. However, it still remains to be understood why the immune response is modulated differently by different liposomal formulations, and which properties are decisive for the outcome. This is a particularly challenging task as it is inherently difficult to isolate the contribution of different properties, as changing one property usually influences one or several others. For instance, when varying the surface charge by altering the lipid composition one may inherently affect other properties, such as membrane fluidity and rigidity, as well as their resistance to enzymatic degradation, etc. Hence, it may be difficult to directly assess the influence of changing different physicochemical properties of carriers on the immune response. Nevertheless, attempts have been made to systematically study the influence on immunogenicity that the physicochemical properties (Figure 4) liposomes used in mucosal vaccines against infectious diseases have, as summarized in further detail below starting with size and morphology (section 5.1), followed by sections addressing zeta potential (section 5.2), antigen localization (section 5.3), liposome resistance to degradation (section 5.4), liposome stiffness (section 5.5), modifications to increase bioavailability (section 5.6) and cell-targeting modifications (section 5.7).

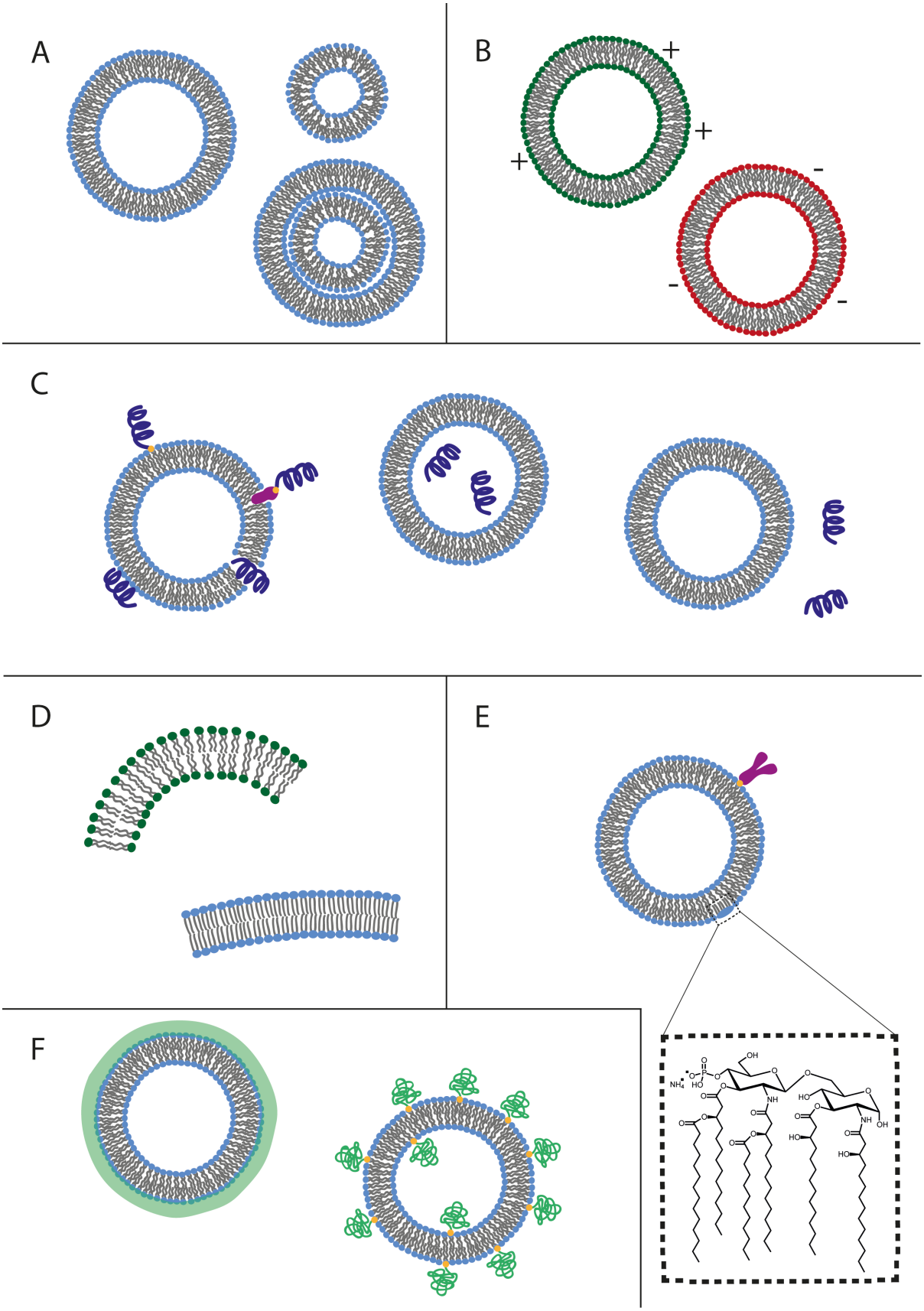

Figure 4. Schematic representations of the physicochemical properties of liposomes that are reviewed here. (A) Liposome size and morphology/lamellarity are difficult to very tightly control but have, in some instances, been investigated. (B) The impact of zeta potential, the difference in electrostatic potential between

the bulk fluid and the slipping plane of the particle, has been extensively examined. (C) The role of the localization of the antigen: on, inside or beside the liposome, has been investigated. There are several modes of antigen association to liposomes. Firstly, antigens may be linked to the surface via covalent attachment or through the use of a hydrophobic anchor that inserts into the membrane. Alternatively, the antigen may associate via adsorption or electrostatic interactions with a particle of opposite surface charge. If the antigen contains hydrophobic regions, these may insert into the lipid membrane and thus couple the protein to the liposome. Furthermore, antigens may be encapsulated in the aqueous core of the liposome. Liposomes may also simply be simply admixed with the antigen. (D) The lipid composition may impact the liposomes' resistance to degradation in biological fluids, as well as their stiffness/bending rigidity and fluidity. (E) Cell-targeting modifications to liposomes to increase their association with cells of interest can be done, for example, by attaching pathogen-associated structural motifs (PAMPs), such as lipid A (in inset, see section 2.2 for further information), or by targeting cell-specific surface proteins using antibodies (e.g. anti-CD103 or anti-DEC205). (F) Modifications aimed at increasing either mucoadhesion or mucopenetration both have the final goal of increasing bioavailability and are commonly done by either fully coating liposomes with polymers or polyelectrolytes, or by adding individually coupled polymers, often some form of poly(ethylene glycol) (PEG).

5.1 Size and morphology

A broad range of liposomes of varying morphologies and with varying sizes (Figure 4A) has been proposed for vaccine delivery and the different particles have been found to induce different effects following mucosal immunization. However, the influence of these parameters on liposome immunogenicity has rarely been systematically investigated and unfortunately details about liposome morphology are not routinely reported. The few studies reporting on the effects of morphology in general, and lamellarity in particular, on immunogenicity show inconclusive results. A comparative study between unilamellar liposomes made from archaeal polar lipids (archaeosomes) with an average diameter of 100 nm and large (on the scale of tens of μm) irregularly shaped liposome aggregates clearly indicated better immunogenicity for the aggregates.[98] Another study reported that oral administration of a "double liposome", consisting of small (~250 nm) liposomes made from SoyPC, dipalmitoylphosphatidylcholine (DPPC), cholesterol and stearylamine encapsulated into a bigger (1 to 10 μm) outer liposome made from dimyristoylphosphatidylcholine (DMPC) and 1,2-dimyristoyl-*sn*-glycerol-3-phosphoglycerol (DMPG), was found only marginally more immunogenic than small liposomes.[111] Additionally, a study using liposomes made from DPPC, DDA and cholesterol with sizes ranging from 70 to 1000 nm for intranasal immunization of mice similarly showed no significant effect of size on immunogenicity.[112]

Constructing homogeneous, strictly monodisperse liposomes of controlled lamellarity is technically challenging; typically, liposomes in a range of sizes and various degrees of lamellarity may co-exist, making interpretations of experimental results difficult. Recent advances in the production of tightly size-controlled liposomes may allow for more accurate comparisons of the influence of size, lamellarity and overall structure in the future.[113]

5.2 Zeta potential

One of the most commonly explored parameters in the context of mucosal vaccine delivery is the charge, zeta potential, of the liposome (Figure 4B). The magnitude of the zeta potential depends on the properties of the particle, but also other factors, such as the ionic strength and pH of the dispersion medium. This must be kept in mind when comparing zeta potential values reported in different studies and under different conditions, as well as when considering the relevance of this parameter in *in vitro* and *in vivo* studies.

Because the cell surface as well as the mucus coating of the mucous membrane is negatively charged, positively charged liposomes will generally exhibit stronger interactions with the cell membrane as well as an increased mucoadhesion.[13, 44] The latter leads to reduced clearance rate, i.e. slower removal from the mucous membranes. This may be beneficial for antigen delivery as both increased interactions with the cell membrane and prolonged exposure time of the antigen at the mucosal surface, are thought to lead to increased cellular uptake of antigen and stronger immune responses.[114, 115] Indeed, cationic liposomes were found to effectively deliver antigen to both mucus and APCs as shown in an in vitro model of the airway epithelium with liposomes made with 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC)/trehalose 6,6-dibehenate (neutral) and DSPC/trehalose 6,6-dibehenate/DDA (positive) with varying amounts of the positively charged DDA.[116] Moreover, cationic liposomes consisting of DOTAP/cholesterol, DMTAP/cholesterol or, most prominently, the polycationic sphingolipid ceramide carbamoyl-spermine (CCS) and cholesterol were shown to effectively stimulate systemic and mucosal humoral and cellular immune responses after intranasal immunizations in mice.[108] In contrast, neutral liposomes with DMPC or anionic liposomes with DMPC/DMPG were comparably ineffective as immunogens.[108] While a positive charge appeared to increase the immunogenicity of liposomes in these cases, this may not always be true. In fact, there are scientific reports suggesting that negatively charged

liposomes are more immunogenic than both zwitterionic and positively charged liposomes and it has even been postulated that anionic liposomes could exert an immunosuppressive effect on alveolar macrophages (a type of macrophage found in the alveoli of the lungs, see chapter 2), and in this way promote an enhanced humoral immune response.[109, 117-120] Hence, it appears that several mechanisms can be modulated by the charge of the liposome. In particular, the influence of charge on immunogenicity may be highly dependent on the administration route, where different microenvironments with varying electrostatic properties may be encountered.

5.3 Antigen localization

There are many ways of incorporating antigens into liposomes. This raises the question whether some strategies are more effective than others in the context of optimizing the immunogenicity of the liposome formulation. Antigens can be hosted in the aqueous core of the liposome, inserted into the hydrophobic part of the membrane or bound to the surface by covalent bonds or intermolecular forces (Figure 4C). Hence, a plethora of combinations exist and those could be used, in combination with various lipid compositions, to enhance resistance against antigen degradation or to facilitate antigen uptake. Thus, the liposome formulation may be tailored for specific needs and purposes. If an oral vaccine is to be designed, one may hypothesize that encapsulating the antigen inside the liposomes is an effective strategy to prevent enzymatic degradation. However, by hiding the antigen in the liposome, the immunogenicity may be compromised because the antigen will not be immediately accessible for APCs. Therefore, choosing how to physically incorporate the antigen in the liposome may have critical consequences on the immune response. Unfortunately, such aspects have not been addressed in a systematic manner thus far. Studies report that when administered orally, encapsulated antigen may more effectively stimulate local IgA and serum IgG antibody responses compared to when soluble antigen is admixed with the liposomes.[121, 122] On the other hand, following intranasal administration, a mixture of antigen and liposomes has been quite effective even compared to liposome-encapsulated antigen.[108, 118] Interestingly, liposomes have been found to exert an immuno-enhancing effect even when administered 48 hours prior to the antigen.[118] Furthermore, surface-bound antigen has been found to be more immunogenic than encapsulated antigen following intranasal immunization.[119] These observations suggest that the intranasal route is less sensitive to antigen degradation compared to the oral route. Thus, depending on the route of administration, it seems clear

that antigens may or may not be immunogenic when exposed, and for many formulations it may, in fact, be advantageous to have a combination of surface-bound and encapsulated antigens. This may also apply to molecular adjuvants; it was found that cholera toxin B-subunit (CTB) adjuvant bound to the surface of the liposome was more effective compared to when encapsulated in the liposome.[123] Furthermore, it has been observed that by altering the lipid-to-antigen ratio, the systemic and mucosal as well as the humoral and cellular immune responses can be differentially induced.[108, 124] Thus, it is likely that the immune response following liposome administration is susceptible not only to the choice of antigen and adjuvant but also to their relative proportions and localization in the liposome.

5.4 Liposome resistance to degradation

The lipid composition (Figure 4D) is known to influence the stability, i.e. resistance to degradation, of the liposome; a more stable formulation might lead to a larger amount of bioavailable antigen and potentially also to a depot, i.e. slow release, effect. Han et al. made liposomes from various combinations of cholesterol, DPPC, dipalmitoylphosphatidylserine (DPPS) and DSPC and found that certain combinations decreased leakage of encapsulated carboxyfluorescein in different solutions simulating conditions in the gastrointestinal tract.[102] Liposomes with DSPC, having a higher transition temperature, were more stable *in vitro* and likely protected antigen better from degradation in the gastrointestinal tract.[102] As aforementioned, using archaeal lipids, liposomes can be made more immunogenic and archaeosomes were found considerably more potent than liposomes made with Egg phosphatidylcholine (EPC)/cholesterol at inducing antigen-specific IgG and IgA antibodies following oral administration in a mouse model.[97] This was attributed by the authors to an increased stability in the gastrointestinal tract and to the fact that the archaeosomes were better retained in the intestine. [97] However, the difference may also partly reflect the fact that the archaeosomes were negatively charged while the EPC/cholesterol-liposomes were neutral and, as discussed in section 5.2, negatively (or positively) charged liposomes are generally more immunogenic than neutral ones.

5.5 Liposome stiffness

Parameters that affect the stability to degradation of liposomal formulations (lipid transition temperature, inclusion of cholesterol) generally also influence the membrane bending rigidity, or deformability (Figure 4D). The role of membrane rigidity on mucosal

vaccination outcomes has only been marginally investigated [108, 125] and studies on the impact of this parameter on the immunogenicity of injected vaccine formulations has had mixed results.[108, 126-134] Complicating investigation of this topic is the fact that, as one approaches nanoscopic length scales, the effective stiffness does not depend only on the membrane rigidity, but also on the membrane strain and internal pressure arising in response to the change in internal volume upon liposome deformation.[91] Moreover, functionalization of the liposome with polymers, antigens and cell-targeting ligands further complicates any prediction of effective liposome stiffness. In addition, experimental quantifications of the stiffness of nanoscopic vaccine liposomes are not trivial (see section 4.4), a fact that stands in the way of systematic investigation of the impact of this property. Indeed, it was in an attempt to address this lack of methods for convenient investigation of liposome stiffness that we undertook the work presented in Paper IV. However, even with accurate quantification of stiffness, predicting its implications on vaccine vector interactions with cells and tissues is not inconsequential. It has been theorized that high liposome flexibility may facilitate cell surface attachment and may, for this reason, be beneficial from an antigen delivery standpoint.[125] Simultaneously, theoretical[135-137] and experimental[131, 137] investigations suggest that high particle stiffness should improve (or decrease[138]) cellular uptake. It would thus appear that liposome stiffness is likely a key factor governing the binding and uptake of liposomes by cells. This prompted us to investigate the influence of this parameter on uptake (Paper III) and antigen presentation (Paper II) by APCs.

5.6 Modifications to increase bioavailability

The microenvironment at mucosal surfaces often promotes a high clearance rate of liposomes. Therefore, various strategies have been tested to enhance mucus penetration or to increase antigen-carrying liposome-to-cell membrane adhesion in order to increase the bioavailability of the vaccine antigens (Figure 4F). Layer-by-layer deposition of polyelectrolytes onto the liposomes, for example, has been used as a liposome-stabilizing approach which resulted in higher specific IgA and IgG antibody levels as well as an increased T cell response.[139] Poly-vinyl alcohol or chitosan have been tested to enhance bioadhesive properties of the liposomes and it has been observed that chitosan-loaded and -coated liposomes, indeed, increased IgG antibody responses.[140] Chitosan is a positively charged polysaccharide that can form strong electrostatic interactions with cell surfaces and mucus and, therefore, increase retention time and facilitate interactions between the

liposome and APCs in the mucous membrane.[141] Additionally, chitosan can transiently open tight junctions between epithelial cells to allow for transmucosal transport.[142, 143] Chitosan-modification of liposomes is accordingly a popular strategy for delivery of peptidic antigens.[140, 144-146] In fact, chitosan-coated liposomes have been shown to give better serum IgG antibody levels compared to liposomes coated with other bioadhesive polymers, such as hyaluronic acid- or carbopol, and host better immunogenicity than uncoated negative, neutral or positively charged liposomes.[145]

Considerable attention has been given to studying how liposomes are retained by and/or taken up across the mucous membranes. Liposome interactions with the intestinal mucosa have been studied *in vivo* and *ex vivo* as well as using various *in vitro* models.[97, 139, 147, 148] The latter models have addressed whether passage of liposomes through the tight junctions of epithelial cells can be achieved. Indeed, tight junctions were reported to be open when using PC/cholesterol-liposomes or liposomes coated with extract from *Tremella fuciformis*.[148] Enhanced immune responses were also observed with mucus-penetrating liposomes made with PEG or the PEG-copolymer Pluronic.[146] Significantly higher specific IgA and IgG antibody levels were found with PEGylated than non-PEGylated liposomes. Modifications with PEG or Pluronic F127 also proved useful in preventing liposome aggregation through steric stabilization to obtain small (< 200 nm) chitosan-coated liposomes. In fact, these shielded chitosan-coated and PEGylated liposomes yielded the highest functional serum antibody titers and the strongest IgA responses of all the formulations tested in this particular study.[146]

5.7 Cell-targeting modifications

In the context of carriers for vaccine delivery, one of the most explored modifications is aimed at targeting the delivery of liposomes to subsets of cells that express a comparatively large number of predefined receptors or binding sites. This is achieved by equipping the liposomes with various targeting elements to increase the number of liposomes delivered to the target cell subset (Figure 4E). For example, targeting components may be added to enhance the uptake by APCs or the penetration of the liposome through the mucous membrane. Additionally, the target receptor may be directly involved in immunological signaling and thereby enhancing of the immunogenicity of the liposomes.

APCs in the mucosal tissues have a high density of surface GM1 and the strongly GM1-ganglioside-binding molecule CTB has been reported to enhance liposome immunogenicity.[101, 149, 150] DCs have similarly been targeted by use of mannosylated lipids or anti-CD40 antibody-coated liposomes, which promoted a stronger immune response.[151, 152] Another popular target on immune cells are TLRs, a type of pattern recognition receptor used by phagocytes to recognize PAMPs, i.e. pathogen-associated structural motifs. For instance, when monophosphoryl lipid A, acting through the TLR4 receptors, was added to liposomes, their ability to stimulate the innate immune response was dramatically improved.[101, 102, 153] Other TLR agonists or *Escherichia coli* heat-labile toxin have also been used in combination with liposomes as adjuvants.[99] Furthermore, liposome linking of CpG, which acts through TLR9 signaling; or *Bordetella pertussis* filamentous haemagglutinin, whose effects include binding to macrophage integrins, have been found to enhance immunogenicity.[154, 155] Targeting macrophages via C-type lectins by galactosylation of liposomes resulted in higher specific IgA and IgG antibody levels compared to unmodified liposomes.[156] Another strategy to target macrophages is to incorporate PS. PS is naturally exposed on the surface of cells undergoing apoptosis and in this way liposomes containing PS may trigger phagocytosis by macrophages. Accordingly, it has been found that liposomes containing DPPS induced stronger IgA responses compared to formulations without DPPS.[153] Combinations of both DPPC/DMPG and DPPC/PS have been found effective at targeting liposomes to macrophages, and DPPC/DMPG was the only formulation to induce a significant antibody response following oral immunization.[121]

Another strategy aims at making uptake through mucous membranes more effective by targeting M cells in the follicle-associated epithelium, the thin epithelial cell layer that is responsible for antigen-uptake from the luminal side. Accordingly, the lectin Agglutinin I from *Ulex europaeus* was shown to improve M cell-mediated transport across the intestinal epithelium.[147, 157, 158] Similarly, liposomes functionalized with antibodies have been found to enhance binding to M cells, and as a result increased levels of IgG, IL-2 and IFN-γ were shown following intranasal immunization.[110]

Many strategies have been proposed to achieve cell-targeting of liposomes, with varying degrees of improved function. A plethora of possibilities can be explored when it comes to

targeting liposomes to the cells of the mucosal immune system. The combination of analytical tools for nanoparticle characterization with suitable *in vitro* and *in vivo* assays will greatly help identify the relative importance of liposome targeting and other properties discussed in this chapter and how they can influence the immune response.

5.8 Concluding remarks

To conclude this chapter, it is clear that the design of a lipid-based vaccine formulation is complex and, needless to say, it is important to consider all of its properties. Thus, liposome size, lamellarity and surface charge as well as lipid composition and rigidity of the membrane can all influence the immune response following vaccination. Importantly, the choice of antigen, with its own inherent physicochemical properties, as well as the position of the antigen and any molecular adjuvant in the liposome affect the function of the formulation. Furthermore, the antigen/lipid ratio and properties of the added adjuvant are also important parameters that change the immunogenicity of the liposome. In addition to this, it should be added that care must be taken when drawing general conclusions based on parallels between different studies, since it is often the case that liposome characteristics other than those directly investigated are inadequately reported. This is important because changing one parameter may completely inverse the effect of another, as specifically discussed in reference [159]. Thus, systematically varying individual properties is indeed a useful approach, but one must not forget that a nanoparticle is characterized by a range of properties and that varying one may influence the biological response to others.

Despite this complexity, it is clear from the literature that lipid carriers can be used to, in a fairly controlled manner, modulate the immune response in a wide variety of model systems. It remains, however, to be elucidated by which mechanism their immunomodulation takes place and therefore, how to tune their properties in order to alter their effect. What are then the ideal properties of a strong and effective lipid-based mucosal vaccine? This question is indeed difficult to answer, not only because the underlying mechanisms remain to be investigated, but also because there is currently no standardized procedure to assess the potential of lipid carriers in the context of vaccination. While specific aspects of the mode of action of liposomes are often studied, for example stability in simulated intestinal fluids, mucoadhesion and APC uptake, more systematic examinations of how different parameters influence different parts of the process remains to be seen. Especially the mechanism behind the immunostimulatory properties of

liposomes, unrelated to their role as carriers, is poorly understood. However, the most fundamental step towards rational design of lipid-based vaccine particles would be to develop a systematic protocol for measuring vaccination outcome as well as for the physicochemical characterization of the particles themselves. Moreover, identifying the immune responses that elicit mucosal protection would aid the rational design of effective mucosal vaccines. One aim of this thesis work is to take steps in this direction.

6
Experimental techniques

The experimental techniques used in this work can be divided into two main categories: techniques used for physicochemical characterization of the lipid nanoparticles and techniques used for characterization of the elicited immune response, in particular uptake and antigen presentation by DCs. The former includes methods for quantification of protein content (section 6.1), particle size determination (section 6.2), examination of particle morphology (section 6.3) and zeta potential (section 6.4). Section 6.5 introduces surface plasmon resonance as a tool to study surface-induced deformation, and thus characterization of the mechanical properties, of liposomes. To characterize the elicited immune response, light microscopy is the technique of choice to follow the details of particle-cell membrane interactions and uptake (section 6.6), while flow cytometry has been used to quantify antigen presentation as well as uptake (section 6.7).

6.1 Fluorometry for protein quantitation

Fluorometry relies on the concept of fluorescence, which is commonly visualized using a Jablonski diagram, as seen in Figure 5A.[76] In fluorescence, a molecule known as a fluorophore has a high likelihood to emit light when excited by incoming light of appropriate wavelength. Light of this wavelength consists of photons with an energy content that corresponds to the energy gap between the ground state (S0) and a higher, excited, state (S1 or S2) of the fluorophore. When such a photon hits the fluorophore, the molecule is excited to the higher energy state. The higher energy state is not stable and hence the molecule rapidly returns to the ground state and in doing so light of a lower energy is emitted, as some energy is lost in thermal processes.[76] The energy, E, of a photon is given by:

$$E = \frac{hc}{\lambda} \qquad (6.1)$$

where h is Planck's constant, c is the speed of light and λ is the wavelength. Hence, the emitted lower energy photon will generally have a longer wavelength. The difference between the peak excitation wavelength and the peak emission wavelength of a certain fluorophore is termed Stoke's shift.[160] A common method used to determine emission spectra for fluorophores and to generally quantify fluorescence is fluorometry. The basic

components of a fluorometer are: a light source, a specimen chamber or sample holder and one or several photodetectors such as photomultipliers and charge-coupled device (CCD) cameras. Additionally, there are commonly monochromators or filters to select specific excitation and emission wavelengths.

Fluorometry can be used to determine protein content either by utilizing inherently fluorescent residues such as tryptophan or by the use of assays in which a fluorescent tag is introduced. The CBQCA assay is one such assay in which the non-fluorescent molecule 3-(4-Carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) reacts with primary amines in the presence of cyanide to form a highly fluorescent derivative (Figure 5B).[88] After acquiring a calibration curve, the magnitude of the emitted fluorescence can be used as a measure of total protein content of a sample.

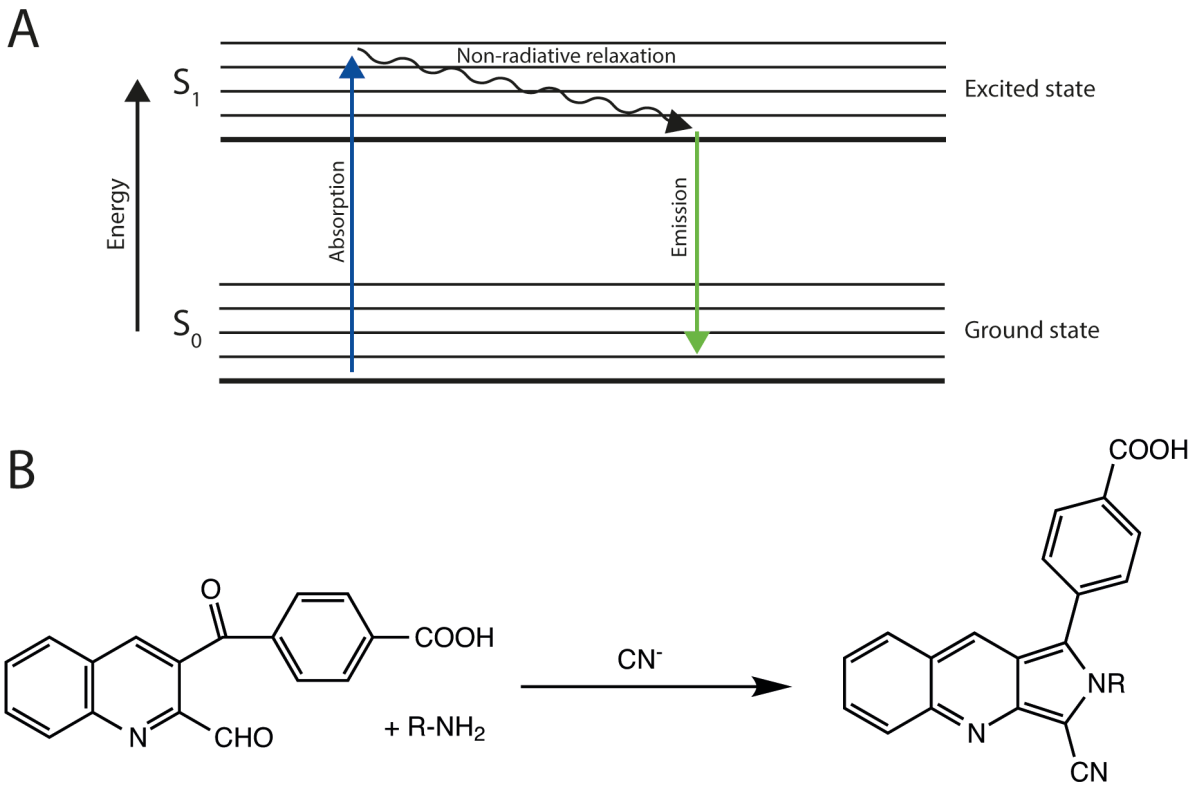

Figure 5. (A) The principal of fluorescence illustrated with a Jablonski diagram. A fluorophore is excited from the ground state S_0 to the excited state S_1 through absorption of light. During relaxation back to the ground state, light of a lower energy is emitted. (B) The reaction of the non-fluorescent molecule 3-(4-Carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) with primary amines in the presence of cyanide to form a highly fluorescent derivative used to quantify total protein content.

6.2 Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) is a nanoparticle sizing and concentration determination technique that relies on visualizing particles in solution undergoing Brownian motion.[70] During a measurement, a laser beam is passed through the solution containing the nanoparticles, which are visualized through light scattering or fluorescence and whose diffusion is recorded using an optical microscope equipped with a camera (Figure 6). Particles in solution undergo Brownian motion in three dimensions. Under the assumption that the motion is uniform in all directions, the captured two-dimensional motions of the particles are tracked by the NTA software in order to determine their diffusion coefficients *D* from the relation:

$$D = \frac{<(x(\Delta t) - x(0))^2>}{4\Delta t} \quad (6.2)$$

where *x is* the mean squared displacement in two dimensions during the time Δ*t*.[161] Assuming spherical particles, the hydrodynamic radius r is given by the Stokes-Einstein equation:

$$r = \frac{k_B T}{6\pi\eta D} \quad (6.3)$$

where k_B is the Boltzmann constant, T is the temperature in Kelvin and η is the solvent viscosity.[70] The hydrodynamic radius, or Stokes radius, of a particle is not its actual physical size but the radius of a hard sphere with the same diffusion rate. This is important to keep in mind when working with non-spherical particles.

NTA can be used for size determination of particles with diameters between approximately 30 to 1,000 nm, with the lower limit being determined by the difference between refractive index of the particles and the surrounding medium.[68]

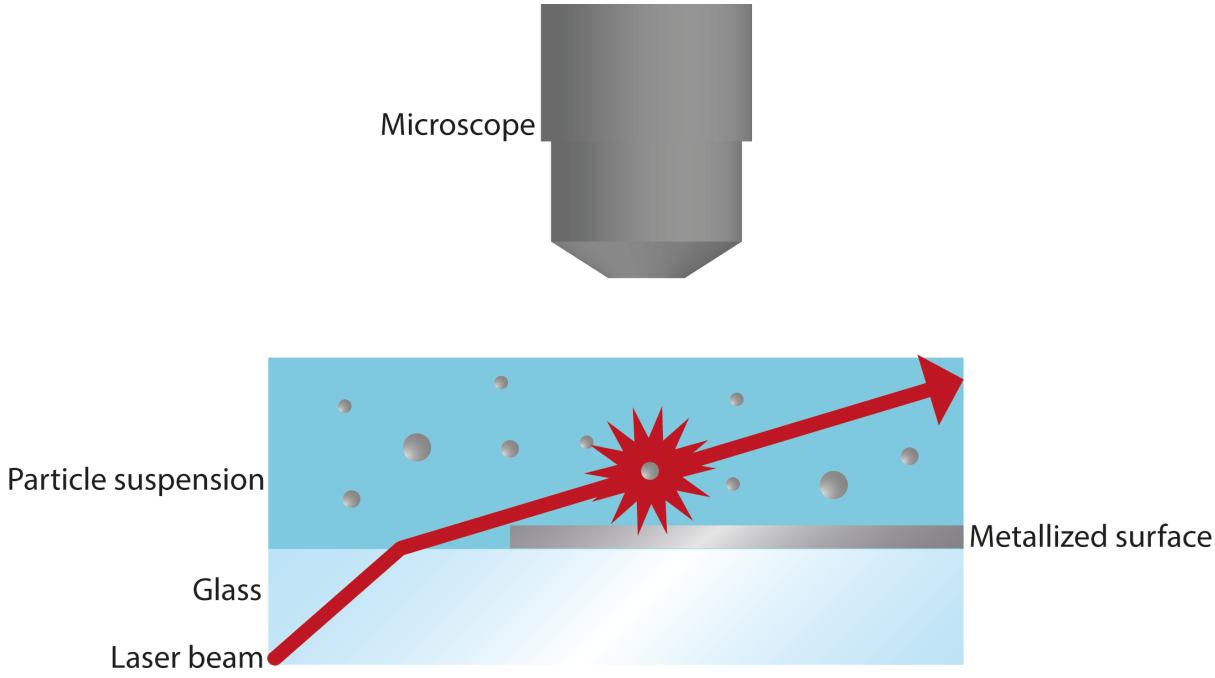

Figure 6. Schematic of a nanoparticle tracking analysis (NTA) setup. Particles undergoing Brownian motion in solution are visualized through scattering of laser light. Their motions are tracked using specialized software in order to determine their diffusion constant and hydrodynamic diameter.

6.3 Cryogenic transmission electron microscopy

Transmission electron microscopy (TEM) is a high-resolution imaging technique in which the image contrast is generated by the scattering of electrons when they encounter structures with high atomic number within the specimen. The instrument consists of an electron source emitting an electron beam, that may be focused onto the specimen with electromagnetic lenses, and a detector, commonly a CCD camera (Figure 7A).[72, 162] The imaging is by necessity performed in vacuum to avoid scattering of the electron beam by air. The analyzed samples are thin, 20-90 nm, and negative staining is often applied to increase the contrast. Negative stains generally consist of a salt of a heavy metal with a high atomic number (42-92) that forms a thin glassy film on top of the specimen.[72] Conventional TEM on hydrated systems is however prone to artifacts due to staining and shrinking due to drying, which affects the perceived morphology of the specimen.[72]

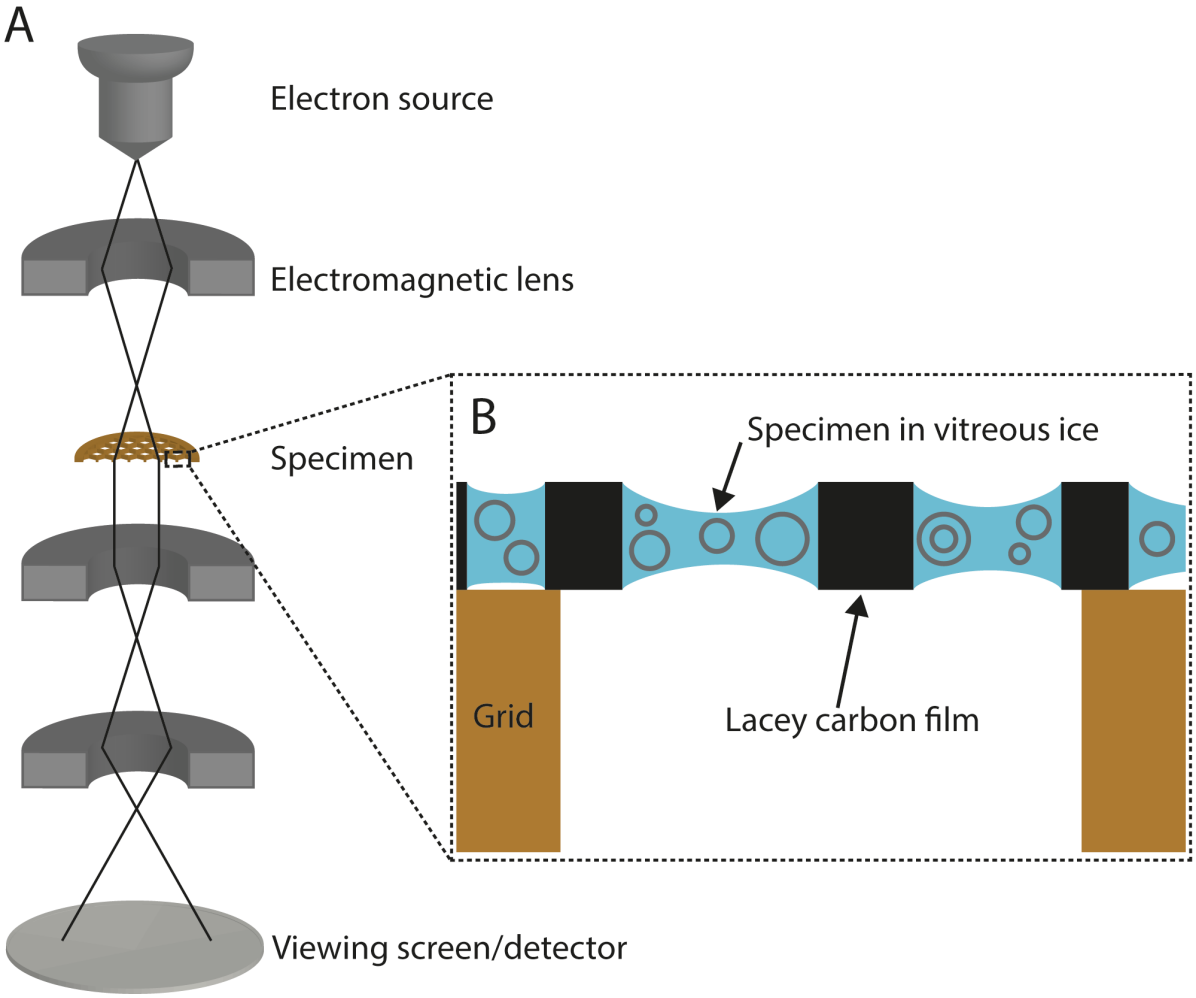

Figure 7. (A) Schematic representation of cryogenic transmission electron microscopy (Cryo-TEM); scattering of electrons is used to visualize structures of high electron density in a cryopreserved specimen. (B) In a common type of cryopreservation, the specimen, in solution, is applied to a lacey carbon film mounted on a copper grid. The supported specimen is plunged into a cryogen leading to formation of vitreous ice, preserving the morphology of fragile structures.

To overcome these limitations, cryogenic TEM (Cryo-TEM) relies on cryopreservation of the sample prior imaging to minimize artifacts. The sample preparation is aimed at creating vitrified specimens that maintains their structural integrity, generally through plunge freezing of thin films or cryo-sectioning of bulk samples.[72] Plunge freezing is a fast and fairly straightforward method in which a small amount of sample in liquid suspension form is added to a supporting substrate, often a lacey carbon film supported by a copper grid. The sample is blotted with filter paper so that only a thin film remains on the substrate and is then plunged into a cryogen with high heat capacity, such as liquid ethane at around -183 °C. Due to the very rapid freezing rate, the liquid suspension vitrifies instead of forming crystalline ice, thus keeping the structures within intact (Figure 7B). The vitrified

samples are transferred to the electron microscope's cryoholder under liquid nitrogen and are viewed at around -173 °C under high or ultra-high vacuum.[72]

Due to the thinness of the film formed through this preparation method, it may be difficult to visualize larger structures. Furthermore, the elements comprising biomacromolecules and polymers, such as proteins and PEG, generally do not scatter enough to provide sufficient contrast to be readily visible with Cryo-TEM.[72] Cryo-TEM of protein- and/or polymer-conjugated lipid particles therefore gives information about the size and morphology of the lipid structures only, unless additional labeling is performed. It is important to note that since Cryo-TEM gives information about the physical size of particles, it can be misleading to directly compare sizes measured from Cryo-TEM images to data obtained with other sizing techniques that measures the hydrodynamic size, such as NTA or dynamic light scattering.

6.4 Laser Doppler electrophoresis

The charge of particles is commonly assessed by measuring their electrophoretic mobility, i.e. their velocity in an electric field.[45] This measure is independent of shape and size and can be assessed using laser Doppler electrophoresis (LDE). An LDE instrument generally consists of a laser providing collimated light that is split into two beams: the scattering beam and the reference beam (Figure 8A).[163] The scattering beam enters the scattering volume, an electrophoretic cell that contains the specimen, a suspension of particles, which scatters the incoming light. The movement of the particles undergoing electrophoresis causes a shift in the frequency Δf of the scattered light compared to the reference beam due to the Doppler effect according to:

$$\Delta f = 2v \cdot \frac{\sin(\theta/2)}{\lambda} \qquad (6.4)$$

where v is the particle velocity, λ is the wavelength of light used and θ is the scattering angle.[163] The light scattered at the angle θ is combined with the reference beam before arriving at the detector. Analysis of the Doppler shift can be made using phase analysis light scattering in order to deduce the electrophoretic mobility of the particles.

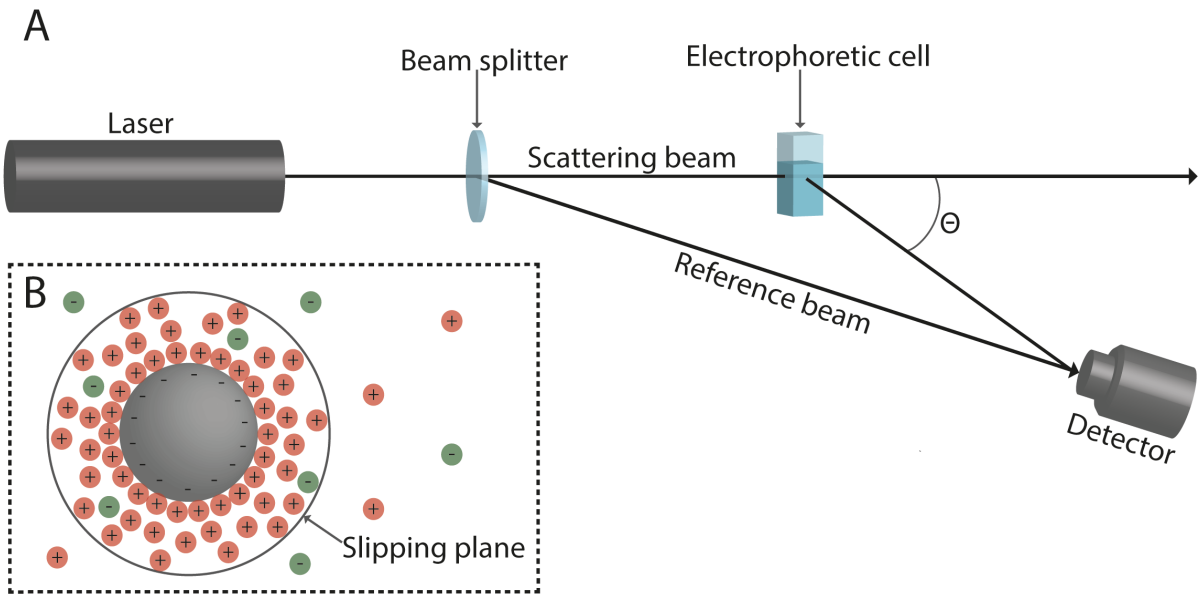

Figure 8. (A) Schematic representation of a Laser Doppler electrophoresis (LDE) instrument; laser light is split into a reference beam and a scattering beam, which is scattered by the particles undergoing microelectrophoresis. The frequency shift between the reference beam and the light scattered at angle θ, due to the Doppler shift is used to deduce the electrophoretic mobility of the particles which is used to determine the zeta potential. (B) The zeta potential is the electric potential, not at the particle surface, but at the slipping plane at the edge of the diffuse electric double layer comprising ions loosely associated with the particle (see section 4.2).

The oscillating electric field applied during LDE causes the particles to move at a velocity that is proportional, not to the charge directly at the particle surface, but rather to the zeta potential, which is the electrostatic potential at the slipping plane at the edge of the diffuse electric double-layer surrounding charged particles (Figure 8B, see section 4.2 for details). The zeta potential ζ can be calculated using the Henry equation:

$$\zeta = \frac{3\mu\eta}{2\varepsilon_0\epsilon_a} f(\kappa a) \qquad (6.5)$$

where μ is the electrophoretic mobility, ε_0 is the permittivity of vacuum, η and ϵ_a are, respectively, the viscosity and the dielectric constant of the dispersion medium and a is the particle radius.[164] $f(\kappa a)$ is Henry's function which depends on the Debye length κ^{-1}. The magnitude of both the Debye length and the zeta potential do not only depend on the particle charge, but also on the ionic strength and pH of the medium in which the particles are dispersed.[45] In cases where the particle radius is much larger than the Debye length ($\kappa a >> 1$), the Henry equation can be simplified using the Smoluchowski approximation of $f(\kappa a) = 1.5$, giving:

$$\zeta = \frac{\mu\eta}{\varepsilon_0\epsilon_a} \qquad (6.6)$$

.[164, 165] At physiological ionic strength, the Debye length is on the order of nanometers, so this approximation is thus often suitable.[45]

6.5 Surface plasmon resonance

Surface plasmon resonance (SPR), a surface sensitive technique utilizing optical excitation of surface charge density waves of free conduction electrons at a metal-dielectric interface, was first described by Kretschmann[166] and Otto[167] in the late 1960s. The technique is commonly used for biosensing following the work of Liedberg et al. in the early 1980s.[168] Commercial instruments often utilize the Kretschmann configuration[166] which, as depicted in Figure 9, consists of a glass sensor slide coated with a metal layer, typically gold, in contact with the ambient medium. The use of a thin enough gold layer enables p-polarized laser illumination through the glass to affect the metal-ambient interface. The dispersion relation for a surface plasmon propagating along this interface can be written as

$$k_{sp} = \frac{\omega}{c}\left(\frac{1}{\epsilon_\mathrm{a}} + \frac{1}{\epsilon_\mathrm{m}}\right)^{-1/2} \qquad (6.7)$$

where ω is the angular frequency, c the speed of light, ϵ_a the dielectric constant of the ambient medium and ϵ_m the real part of the complex dielectric function of the metal. Excitation of the surface plasmon occurs when $\epsilon_\mathrm{m} < 0$, $\epsilon_\mathrm{a} > 0$ and the portion of the incident light wave vector that is parallel to the interface is of equal magnitude to k_sp. By using light incident to the sensor at an angle θ, at which a small part of the light gives rise to an evanescent wave penetrating outside of the glass (see section 6.6.2), the wave vector parallel to the surface of the incident light is

$$k_x = \frac{\omega}{c}\sqrt{\epsilon_g}\,sin\,\theta \qquad (6.8)$$

where ϵ_g is the dielectric constant of the glass prism. During an SPR experiment, the angle of incidence is varied and at a certain angle, known as the resonance angle, k_x is equal to k_sp and surface plasmon resonance occurs, manifesting as a minimum in the reflected light intensity. Given that $|\epsilon_\mathrm{m}| \gg |\epsilon_\mathrm{a}|$, equation (6.7) can be simplified to $k_\mathrm{sp} = \frac{\omega}{c}\sqrt{\epsilon_\mathrm{a}}$ and further to $k_\mathrm{sp} = \frac{\omega}{c} n_\mathrm{a}$ where n_a is the refractive index of the ambient medium. Thus, shifts in the resonance angle can be used to probe, with high sensitivity, changes in refractive index close to the interface, for example due to adsorption of molecules or changes in the conformation of particles bound to the surface.

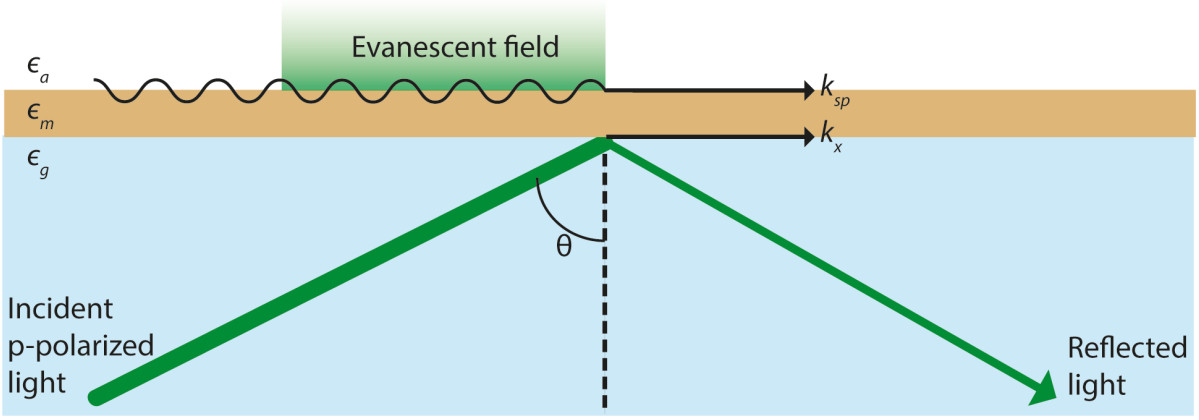

Figure 9. Schematic representation of the key theoretical principles behind surface plasmon resonance (SPR) using the Kretschmann configuration. ϵ_m is the real part of the dielectric function of the metal, ϵ_a and ϵ_g are the dielectric constants of the ambient medium and the glass, respectively. k_{sp} *and* k_x are the wave vectors of the plasmon and the horizontal component of the p-polarized light incident at an angle θ, causing total internal reflection and an evanescent wave. When $k_x = k_{sp}$ resonance occurs, electrons at the gold-ambient interface oscillate coherently. k_{sp} is dependent on the refractive index of the ambient medium, which can be measured as a function of a measurable decrease in the amount of reflected light at the critical angle.

The SPR response R, upon molecular binding to the gold surface can be described using the expression

$$R = S(n_f - n_a)\left(1 - e^{\frac{-d}{\delta}}\right) \qquad (6.9)$$

where S is the sensitivity factor of the system, n_f and n_a are the refractive indices of the bound film and the ambient medium, respectively, δ the decay length of the light intensity, and d corresponds to the thickness of the bound layer, e.g. the height of bound vesicles.[96, 169] Simultaneously, the surface coverage Γ, in mass per surface area, is described by

$$\Gamma = \frac{(n_f - n_a)}{dn/dc} d \qquad (6.10)$$

where dn/dc is the derivative of the refractive index with respect to the molecular concentration in solution.[170] Together, equation (6.9) and (6.10) yield the expression

$$\Gamma = \frac{d}{S(dn/dc)[1 - e^{-d/\delta}]} R, \qquad (6.11)$$

which allows for calculation of the surface coverage Γ from the SPR response R. By probing with two wavelengths of light simultaneously (multiparametric SPR), the ratio of the SPR responses R can be expressed as

$$\frac{R_{\lambda_1}}{R_{\lambda_2}} = \frac{S_{\lambda_1}(dn/dc)_{\lambda_1}\left(1 - e^{-\frac{d}{\delta_{\lambda_1}}}\right)}{S_{\lambda_2}(dn/dc)_{\lambda_2}\left(1 - e^{-\frac{d}{\delta_{\lambda_2}}}\right)} \qquad (6.12)$$

where λ_1 and λ_2 denote the two wavelengths of light used. This relation can be used to determine the thickness d of a bound layer.

6.6 Light microscopy

Methods capable of identifying and quantifying cellular responses are useful when assessing how particle properties affect key events in the immune response. In the context of vaccination key events include antigen uptake by an APC. Uptake processes can be characterized and quantified using microscopy and current high-resolution imaging methods provide opportunities to do so with precision. In particular, live-cell imaging allows us to observe the dynamics of such processes in real-time. In this chapter, a brief overview of the basics of optical microscopy, some specialized microscopy techniques and examples of how they have been used to study cellular uptake of different types of particles in sizes ranging from tens to a few hundred nm will be given.

Convex lenses have been used for more than five hundred years to magnify objects beyond the human eye's ability to observe and the invention of the microscope took place in the 17th century.[171] Thanks to these advances, we have been able to visualize and understand microorganisms, our own cells and eventually also their constituents.

There are many microscopy subtypes and setups but the basic construction of an optical microscope is mostly the same in all cases. In essence, light from a light source such as a lamp, LED or laser passes through a condenser (not necessarily when a laser is used) and then interacts with a specimen.[171] The light is collected by the objective, which contains one or several lenses and that helps focus the image of the specimen at the optical plane of an eyepiece or a camera.[171] In the simplest form of microscopy, brightfield, the light is simply transmitted through the specimen; all the light is collected and the contrast is given by the attenuation of the light due to the sample. However, many specimens have low contrast, making structures difficult to separate from the background. Therefore, there are variants of optical microscopy aimed at enhancing the contrast, such as darkfield, phase contrast, polarization microscopy and differential interference contrast (often referred to simply as DIC).[172-175] In addition to the contrast, the resolution is a critical parameter for how well small objects are visualized using light microscopy. The resolution limit is a constraint that the wavelike properties of light place on conventional light microscopy. When light from a point source passes through a circular aperture, such as a lens, it is

diffracted and will appear as a bright spot surrounded by a series of concentric circles (an Airy disk and Airy pattern).[176] When two objects come close together, their Airy patterns overlap and they cannot be visually separated. The work by Abbe, Rayleigh and Airy helped formulate the dependency of the resolution limit, *d*, on the wavelength of the light, λ, and the objective used for imaging:

$$d = \frac{1.22\lambda}{2n\,sin\theta} = \frac{1.22\lambda}{2NA} \quad (6.13)$$

where *n* is the refractive index of the medium, θ is the half angle subtended by the objective and *NA* is the numerical aperture of the objective.[176-178] For modern instruments, this means approximately half the wavelength of the light, in practice.

An important component of many modern microscopy techniques is the use of fluorescent labels, such as antibodies conjugated to fluorophores (see section 6.1) or proteins which are expressed with the tag directly attached. This has been crucial not only to detect objects smaller than a couple hundred nanometers but also to visualize cellular structures and observe dynamic processes, such as uptake. Fluorescence has been instrumental in the development of the imaging field, both as a means to improve the contrast but additionally in allowing us to move beyond the diffraction limit using techniques such as confocal laser scanning microscopy and super-resolution imaging.

6.6.1 Widefield fluorescence microscopy

The simplest type of fluorescence microscopy is widefield fluorescence microscopy, or epifluorescence microscopy. In this technique, multichromatic light is sent through an optical excitation filter that allows only light of wavelengths suitable for excitation of a particular fluorophore to pass; the light meets the main dichromatic mirror, a component that selectively reflects or transmits light of different wavelengths. The dichromatic mirror reflects the short-wavelength excitation light through the objective and onto the specimen. The light emitted by fluorophores in the sample is collected by the objective and meets the main dichromatic mirror, which transmits this light that then passes through an emission filter that allows light of only certain wavelengths to pass to the eyepiece or camera (Figure 10).[179] The excitation and emission filters together with the dichromatic mirror are generally mounted into a filter cube. It is common to have multiple filter cubes suitable for imaging different fluorophores. One can then switch from one to the other and image different labeled structures in sequence.[180]

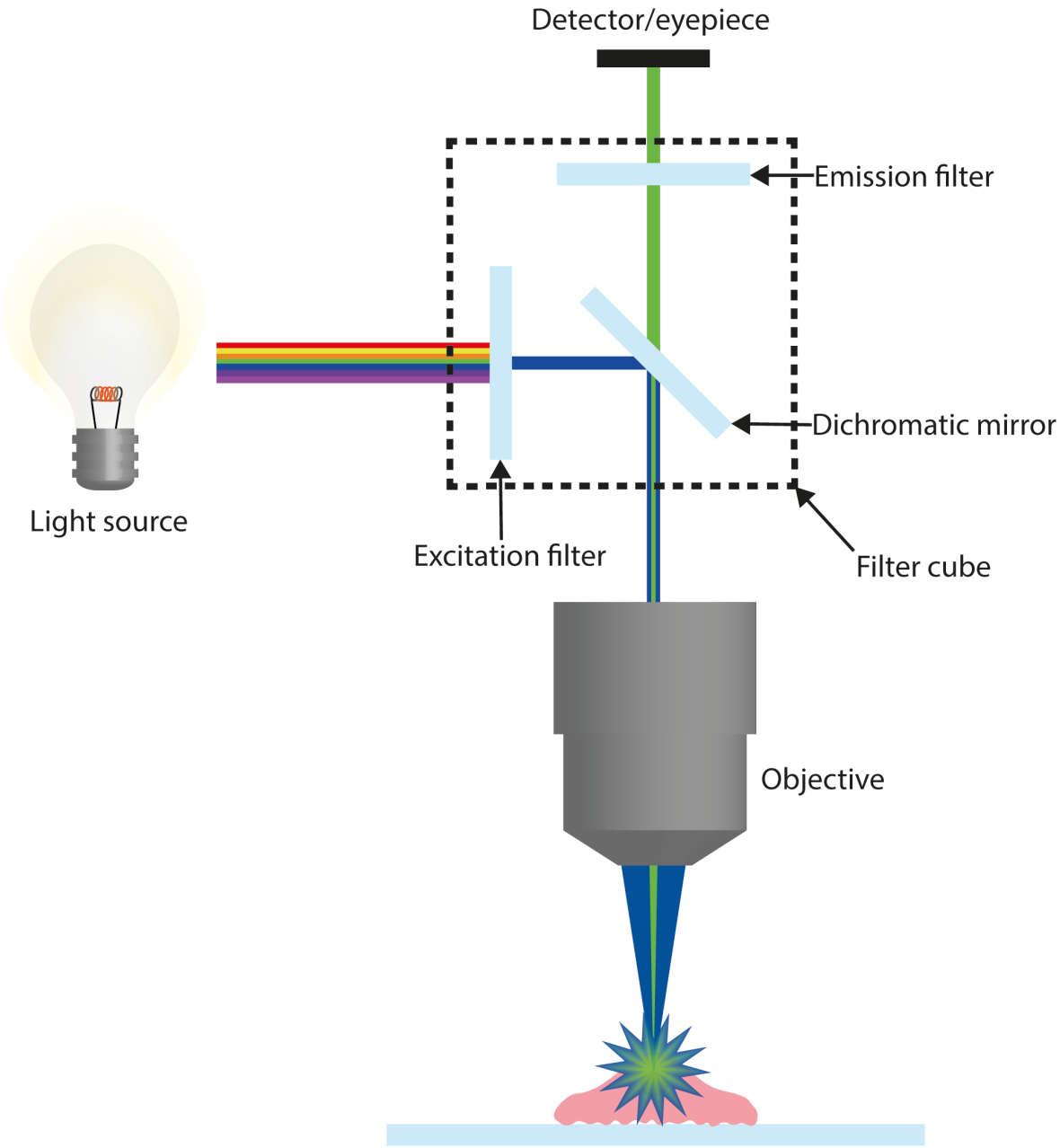

Figure 10. General setup of a widefield fluorescence microscope, illustrating the light path from the light source, through excitation filter, dichromatic mirror and objective to the sample, where it excites a fluorophore that emits light of a different wavelength. The emitted light travels through the objective, dichromatic mirror and emission filter to the eyepiece or detector.

Widefield fluorescence microscopy has been applied to study cellular uptake of different particles of interest. Van der Schaar et al. used widefield microscopy to study cellular uptake of DiD-labelled Dengue virus.[62] They elucidated both the clathrin-mediated internalization process and the endocytic trafficking of the virus through fluorescent labeling of endocytic machinery components.[62] Using HIV-1 virus-like particles, Endreß et al. showed two types of HIV-cell interactions: either the virus-like particle became

immobilized upon contact or there was a very short-lived dynamic interaction (in the range of 20-50 ms) followed by dissociation.[181] de Bruin et al. used epidermal growth factor to direct delivery of polyethylenimine polyplexes to cancer cells that overexpress epidermal growth factor receptor. Widefield microscopy revealed faster and more efficient internalization of epidermal growth factor receptor-targeted compared to untargeted polyplexes.[182] Furthermore, Tian et al. studied cellular uptake and processing of exosomes.[60] Single particle tracking was used to study the movement of exosomes in medium, on the cell surface and intracellularly in endosomes and lysosomes, identifying distinct movement patterns distinguishing membrane-bound from freely diffusing exosomes. Trypan blue was used to distinguish between intra- and extracellular DiI-labelled exosomes. Octadecyl rhodamine B chloride-labelled exosomes were used to discern that exosomes were taken up through endocytosis and not fusion with the cell membrane.[60]

A drawback of widefield fluorescence microscopy is that since all emitted light within a wavelength range is collected, there is usually a high background from out-of-focus fluorophores. Total internal reflection fluorescence (TIRF) microscopy and confocal laser scanning microscopy (CLSM) are two techniques that have been developed in order to address this issue.

6.6.2 Total internal reflection fluorescence microscopy

TIRF microscopy is a surface sensitive technique that relies on selectively exciting fluorophores using an illumination depth restricted to the area closest to an interface between two materials of different refractive indices, n_1 and, n_2. The fluorophores that are outside of the illuminated area remain unexcited and do not contribute to the background fluorescence. The materials of the interface are generally a sample, with low refractive index, e.g. water, on top of a glass coverslip, with high refractive index, as seen in Figure 11.[183]

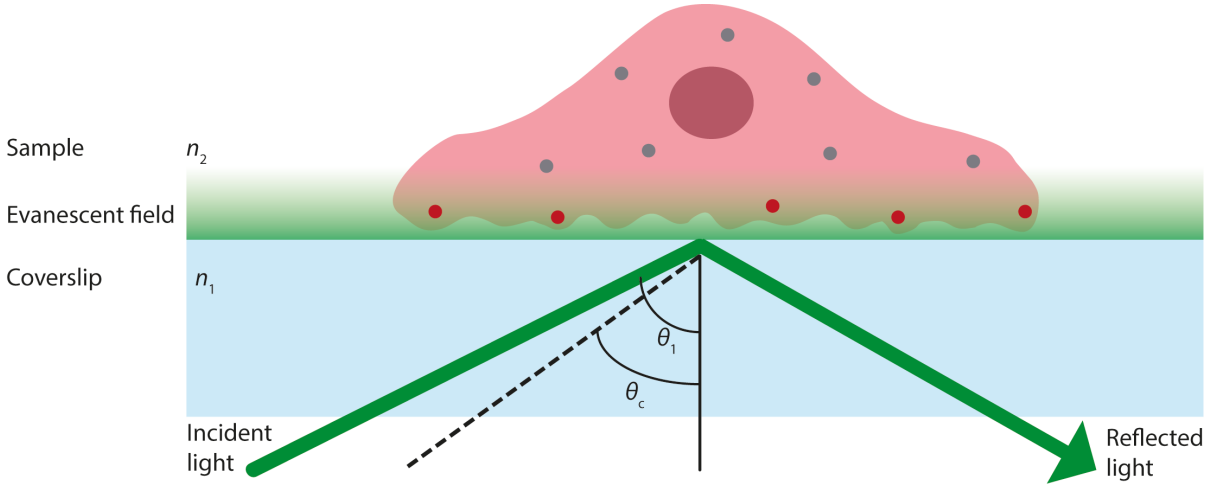

Figure 11. Schematic of total internal reflection fluorescence (TIRF) microscopy. When incident light of angle θ_1, greater than the critical angle θ_c, encounters an interface between a medium with high refractive index (n_1, coverslip) and a medium with low refractive index (n_2, sample), the light is totally internally reflected. This leads to the formation of an evanescent field that extends a small distance into the sample. Thus, only the fluorophores within this field emit fluorescence while fluorophores that are further away from the interface remain unexcited.

How a light beam is refracted when passing through such an interface is described by Snell's law:

$$n_1 \sin\theta_1 = n_2 \sin\theta_2 \qquad (6.14)$$

where θ_1 is the angle of incident light and θ_2 is the angle of refracted light. If the second material has a lower refractive index than the first ($n_1 > n_2$) and the angle of incidence is equal to what is termed the critical angle, θ_c, the refracted light will travel along the interface of the two materials. The critical angle is given by:

$$\theta_c = \sin^{-1}\frac{n_2}{n_1}. \qquad (6.15)$$

If the angle of incidence is larger than the critical angle ($\theta_1 > \theta_c$), the light is totally internally reflected at the interface, giving rise to an evanescent field that extends a small distance into the second material.[183] The light intensity, I, decays exponentially with the distance from the interface, z, according to:

$$I(z) = I_0 e^{-z/d} \qquad (6.16)$$

where I_0 is the intensity at the interface and d is the characteristic decay depth, defined as:

$$d = \frac{\lambda}{4\pi n_2}\left(\frac{\sin^2\theta}{\sin^2\theta_c} - 1\right)^{-1/2} \qquad (6.17)$$

where λ is the wavelength of the incident light.[184] The surface-confined illumination eliminates background fluorescence, making TIRF microscopy a technique with high signal-to-noise ratio compared to widefield microscopy.

TIRF microscopy has been used to study cellular uptake, for example by Schmidt et al., who studied pH-triggered fusion of vaccinia virions to HeLa cells using a microfluidic cell trap.[64] The viruses were labelled with a self-quenching concentration of octadecyl rhodamine B chloride and the virus cores were GFP-tagged. A reduction of the pH was used to induce fusion, which caused dequenching of the octadecyl rhodamine B chloride simultaneous to reduction of the GFP signal due to internalization of the virus core.[64] TIRF has also been used to visualize quantum dot diffusion on the surfaces of immune cells, with single particle tracking used to determine diffusion constants, followed by uptake quantified by measuring the overall decrease in fluorescence as the quantum dots disappeared from the field of view when taken up.[185] It was shown that the size and shape of the quantum dots influence both their final intracellular fate and their behavior on the cell membrane; more specifically, quantum dots with a high aspect ratio exhibited slower movement.[185] Furthermore, TIRF has been used to show the dynamics of cholesterol-modified Cy3-labelled siRNA internalization.[186]

6.6.3 Confocal laser scanning microscopy

CSLM is a popular technique that allows optical sectioning, which is achieved through the use of laser illumination, providing an intense and focused illumination spot, and a pinhole, an adjustable aperture in the conjugate focal plane.[187] The pinhole allows only light from the focal plane to pass, thus achieving optical sectioning, while additionally eliminating light from the Airy pattern of the illumination spot.[179] That the point spread function is reduced in this manner leads to an improvement in the lateral resolution $d_{x,y}$:

$$d_{\mathrm{x,y}} = \frac{0.4\lambda}{NA} \qquad (6.18)$$

where λ is the wavelength of light and NA is the numerical aperture of the objective used.[180] The image is formed by scanning the illumination over the specimen while recording the emitted intensity spot by spot (pixel by pixel) with a photomultiplier tube, which is ideal for detecting weak signals.[187] By axially moving the focal plane, image stacks can be collected in order to obtain three-dimensional information. Axially, the resolution limit d_z is given by:

$$d_z = \frac{1.4\lambda n}{NA^2} \quad (6.19)$$

where *n* is the refractive index and the other parameters as previously defined.[180]

CLSM is a useful technique when studying cellular uptake and trafficking, since optical sectioning can be used to provide three-dimensional information, something that is not obtainable with widefield microscopy. In continuation of van der Schaar's work[62] mentioned in the widefield section, Chu et al. showed, using confocal microscopy, that interaction takes place between DiD-labelled Dengue virus and GFP-tagged autophagosomes during early Dengue infection.[63] CLSM has been widely used for studying uptake of liposomes for pharmacological applications both *in vitro* and *in vivo*.[188] There are essentially four ways by which liposomal content can be delivered: extracellular release of content following adsorption to the membrane, endocytosis, fusion of the liposomal and cell membranes and exchange of lipophilic compounds from the liposomal to the cell membrane.[188] By using fluorescent labeling in a strategic manner, confocal microscopy can be used to determine which mechanism is involved in a particular case. For example, by loading self-quenching concentrations of a dye inside the liposome and observing where dequenching occurs, it can be observed where the cargo is released.[188] Labeled lipids can be used to elucidate whether uptake takes place through fusion or through an endocytic pathway by observing co-localization with certain labeled cell membrane constituents.[188]

6.7 Flow cytometry

An important step of the immune response is the antigen presentation by DCs, which is a crucial step for activation of T cells. The amount of antigen presented on the DC surface in response to changes in the physicochemical properties of vaccine vectors can be assessed using flow cytometry. Indeed, flow cytometry is often used for characterization and quantification of cells and cell constituents. It is a popular technique in different fields of research and has a set of uses ranging from, for example, determination of cell viability to quantification of phagocytosis.[189, 190] In recent years, flow cytometry has, in addition to being an invaluable research tool, become an important diagnostic and prognostic tool in the clinical treatment of cancer and immunological diseases.[191] Furthermore, the use of flow cytometry has stretched beyond the analysis of cells to also include biologically relevant micro- and nanoparticles, such as exosomes.[192]

The large variety of applications and their increasing complexity aside, the flow cytometry technique relies, in essence, on three basic building blocks: 1) the arranging of the cells into a single file through a flow cell, 2) the optical system comprising one or several lasers generating the illumination of the sensing volume through which the cells pass, as well as a set of lenses and filters to focus and direct the light which is scattered and/or emitted by the cells, and 3) the electronics used to convert this light to an electronic signal. Due to the different components comprising a flow cytometer, one might say that there is not a singular origin of the technique, but rather several advancing paths converging into the development of cell microfluorometry by van Dilla et al. in 1965.[193] This chapter will give a brief overview of the theory behind flow cytometry, describing a typical instrument and the physical phenomena it employs.

6.7.1 Principles and instrument design

As previously touched upon, a flow cytometer can be said to consist of three main components: a microfluidic system, an optical system and an electronic system that function in a synchronized manner (Figure 12).[194] In this section, each of these systems is presented in some detail.

6.7.1.1 Microfluidics

The role of the microfluidic system is to ensure that the cells pass in a controlled manner one by one through the sensing volume, or interrogation point as it is sometimes referred to. This is crucial to maintain uniform and reproducible illumination conditions. The arrangement of cells into "a single file" is achieved through hydrodynamic focusing by use of a sheath flow. A pressurized stream of sheath fluid is maintained into which the sample is injected at a higher pressure, causing a difference in flow speeds between the two fluids that maintains the cells in the central stream, the "core". A strategic narrowing of the flow cell causes a simultaneous increase of the flow speed and a decrease of the core cross-section to the point where cells generally pass through the sensing volume one by one.[195] Generally speaking, it is crucial to maintain laminar flow by avoiding e.g. too acute narrowing of the flow cell and sharp edges in the design as well as blockages and air bubbles during operation. The nature of the flow in a tube is determined by four parameters, whose relationship is often expressed by the dimensionless Reynolds number, *Re*:

$$Re = \frac{vd\rho}{\eta} \qquad (6.20)$$

where v [m/s] is the average velocity throughout the cross-section of the tube, d [m] is the tube diameter, ρ [kg/m^3] and η [kg/sm] is the fluid density and viscosity, respectively. At *Re* greater than 2,300 the laminar flow starts to break down in favour of turbulence.[196] The sensing volume is generally located in an area with "slug flow", characterized by constant flow speed across the diameter of the core, a result of the aforementioned narrowing of the flow cell. This is advantageous as it minimizes the velocity differences between cells at different distances from the centre of the core compared to the parabolic flow profile of a laminar flow that arises from the no-slip boundary condition.[195]

6.7.1.2 Optics

When cells pass through the sensing volume, i.e. the laser beam, the light that is scattered, and emitted in the case of fluorescently labeled cells, gives information about their properties. Physically speaking, scattered light consists of light that has been diffracted, reflected, refracted, anomalously diffracted and Rayleigh scattered.[196] Maxwell's equations can be solved to describe the propagation of light after it has been scattered by an object. In the case of a cell, there are many intracellular objects with varying properties, and the morphological and biochemical complexity of a cell thus make the mathematical description of this process challenging even though it has been attempted.[197-201] Such modeling is however not standard in flow cytometry, where the light scattering properties of cells are measured and related to two main properties. Light that is deflected around the edges of the cells, parallel to the direction of the laser beam, is termed forward scatter (FSC) and gives information about the size of the cells. Light that is scattered perpendicularly to the laser beam is termed side scatter (SSC). Side-scattered light is primarily scattered from intracellular structures, therefore giving information about the internal complexity.[190] The intensity of the SSC is proportional to what is often termed cell granularity.

In addition to the information from the scattered light, which is obtained label-free, features of interest can be tagged with fluorescent labels (see section 6.1). Fluorescent probes are utilized in order to detect and quantify the amount of for example nucleic acids, proteins or to assess cell viability.[190] Since different fluorophores have different emission and excitation spectra, a cell can be stained with several probes with different fluorophores (fluorochromes). The most common fluorophores used for flow cytometry include fluorescein isothiocyanate (excitation/emission 495/520 nm), phycoerythrin (excitation/emission 565/578 nm) and allophycocyanin (excitation/emission 650/660

nm).[190] Once excited by lasers with light of suitable wavelengths, monochromatic mirrors and optical filters are used to guide emitted light from different fluorochromes in different "channels" to different detectors.[195]

6.7.1.3 Electronics

The electronic system in a flow cytometer consists of a series of detectors that convert the scattered and emitted light into electrical currents that can be recorded and visualized. There are generally two types of detectors used: photodiodes and photomultiplier tubes. Photodiodes are typically used for the FSC, which has a high intensity, while photomultiplier tubes are used to amplify and detect SSC and emitted light.[202] In both cases, the output signal is proportional to the incoming number of photons.[190]

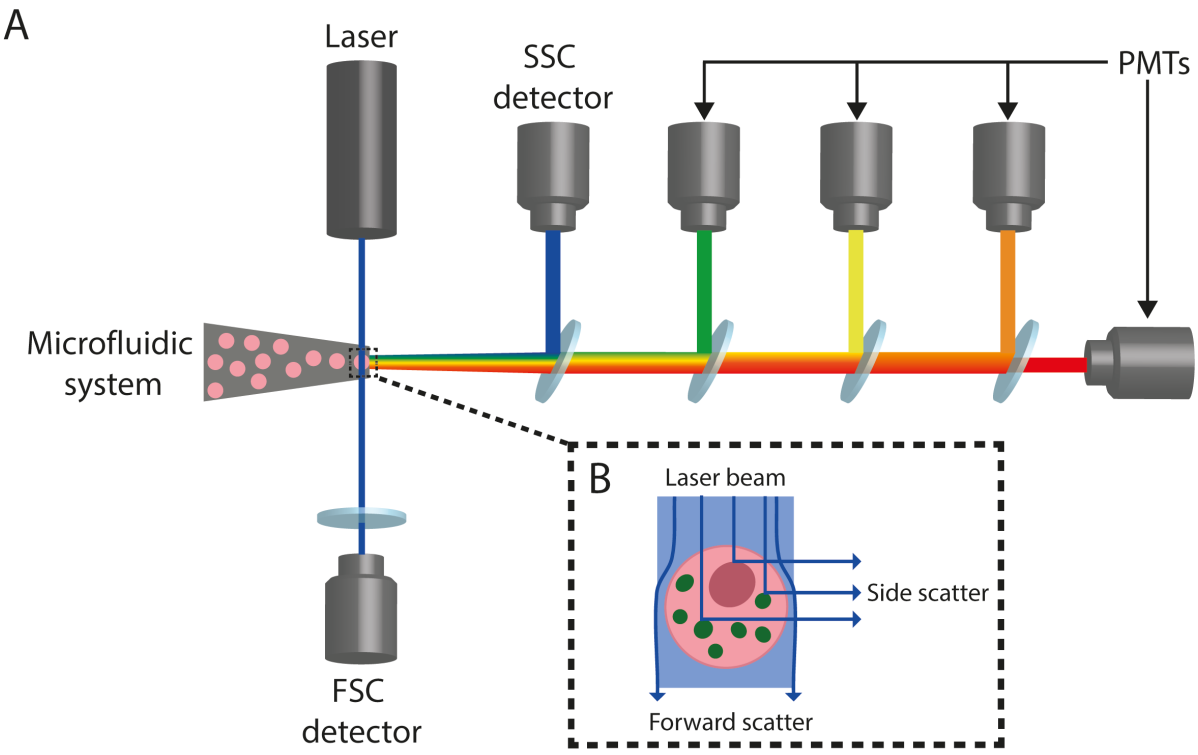

Figure 12. (A) A schematic representation of a flow cytometer. The microfluidic system arranges the cells into a single file through the beam of one of several differently colored lasers, i.e. the sensing volume, (B). The light that is scattered parallel to the laser beam, termed forward scatter (FSC), provides information about the size of the cells and the light that is scattered perpendicularly, termed side scatter (SSC), gives information about the internal complexity of the cells. Dichromatic mirrors and optical filters are used to guide the SSC and light emitted from fluorescent labels to different photomultiplier tubes (PMTs).

6.7.2 Data analysis

In flow cytometry many parameters are usually measured on numerous individual cells, which means that a large and important part of the method comprises the processing of the data. Common ways to display the data include dot plots, which visualize the magnitude of two parameters at the same time, and histograms, which visualize the number of cells and to which degree they are, for example, expressing a marker of interest.

6.7.2.1 Spectral compensation

Spectral (or colour) compensation is a standard procedure used when several fluorochromes are used in one measurement. Overlap of the emission spectra of the fluorophores means that light from several fluorochromes can pass through the emission filters and be detected in the same channel. Spectral compensation is thus needed to adjust for the light each fluorochrome contributes with in channels other than its own, and is done by measuring each fluorochrome alone and calculating how much of the total light each contribute with to the different channels.[190] This information is then used to compensate for any overlap in the subsequent measurement.[203]

6.7.2.2 Gating

Gating is a process commonly used to eliminate results from debris and dead cells and to subsequently enumerate cells with certain properties.[190] Cells are visualized in a two-dimensional scatter plot in which the user defines one or several regions, restricting further analysis to certain subsets of cells. By in this manner iteratively visualizing and zooming in on cells with a certain set of markers, identification and quantification of cell subpopulations is performed.[204]

7
Results

The general scope of this thesis work has been to gain fundamental understanding of how the physicochemical properties of lipid nanoparticles used as mucosal vaccine carriers influence the development of protective immunity and to reveal the properties that are decisive for effective vaccine delivery. Such knowledge will, without doubt, help rationalizing the process of designing vaccine formulations. This goal requires characterization of both the vaccine particles and the biological response they elicit, and in this work, we have made an attempt to undertake both. We have addressed the biological response to antigen-carrying lipid particles using model systems of varying levels of complexity, ranging from an *in vivo* immunization model to simpler, yet informative, *in vitro* models. As for particle characterization, we have identified a need for protocols for physicochemical characterization of nanoparticles relevant in this context; it has been our goal to contribute to the field by developing methods to characterize the stiffness and optical properties of small vesicles.

In this chapter, I provide brief summaries of key findings of the appended papers, as well as some additional experimental results of relevance for the scope of the thesis work. In Paper I, we performed a detailed characterization of the immunogenicity of two types of antigen-carrying liposomes in an *in vivo* mouse model and identified that the liposome formulation most capable of efficiently inducing a local $CD4^+$ T cell response was also the most protective against disease. In Paper II, we therefore focused on optimizing T cell priming ability by studying antigen presentation by, and activation of, dendritic cells *in vitro*. During that particular work, we discovered that gel-phase liposomes were more immunogenic vaccine carriers than those with fluid phase membranes. In an attempt to better understand the mechanisms underlying this effect, we studied uptake of gel and fluid phase liposomes in Paper III. As an extension, we present additional experimental results from an exploration of single-cell-and-particle imaging for the investigation of the interaction between vaccine carriers and the outer cell membrane of dendritic cells. Additionally, we also present further investigations of the immunogenicity of formulations investigated in Paper II and III. As for particle characterization, in Paper IV, we used SPR

in combination with cell membrane mimics to characterize liposome deformation induced upon specific ligand-receptor pairing between the liposome and cell-membrane mimic. Different degrees of deformation and binding valency were observed for gel and fluid phase liposomes, which might be of relevance for differences observed in cellular uptake. Finally, in Paper V, we present how a newly developed waveguide-based microscopy setup can be used to characterize biological nanoparticles modified with a dye that shares similarities to the one used to visualize cellular uptake in Paper III.

7.1 Paper I

In Paper I, we aimed at combining the universal influenza A vaccine candidate CTA1-3M2e-DD with liposomes into an effective mucosal vaccine formulation. CTA1-3M2e-DD is a fusion protein that combines the mucosal adjuvant CTA1-DD with the ectodomain of influenza matrix protein 2 (M2e), which is highly conserved in all human influenza A virus strains. In addition to three repeats of M2e, the fusion protein consists of the enzymatically active cholera toxin A1 subunit (CTA1), which has an immunomodulating effect, and a dimer of the D-fragment from *Staphylococcus aureus* protein A (DD): an effective DC targeting moiety. In this work, the fusion protein was formulated into two types of POPC-based liposomes: non-PEGylated, where fusion protein was covalently attached directly to functionalized lipid headgroups, and PEGylated, where it was attached at the ends of PEG(2000) spacers (Figure 13).

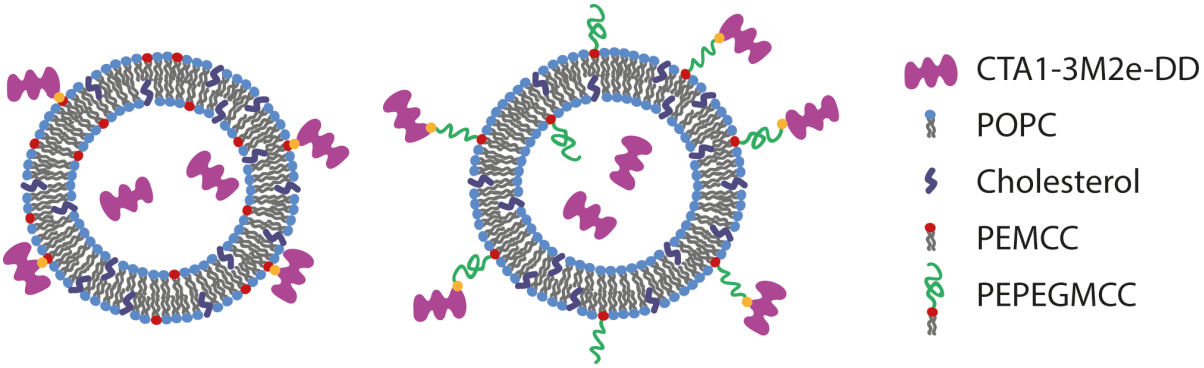

Figure 13. Schematic of the non-PEGylated and PEGylated vaccine vectors used in Paper I. The liposomes where 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)-based and contained 10% cholesterol. The fusion protein was encapsulated and covalently attached using thiol-maleimide chemistry, either directly to lipid headgroups (non-PEGylated) or to the ends of PEG(2000) spacers (PEGylated).

The liposomes were characterized with respect to size, morphology, protein load and surface charge. The main differences between the two formulations, in terms of physicochemical characteristics, besides the PEGylation, were a difference in zeta

potential, the non-PEGylated liposomes being more negatively charged, and protein load; the PEGylated carried approximately 30-40% more proteins.

A thorough *in vivo* investigation of the immunogenicity of fusion protein-carrying liposomes compared to free fusion protein revealed several interesting differences. Upon intranasal immunization, the non-PEGylated vector lead to significant protection from a lethal challenge with a live heterosubtypic influenza virus (a virus of a different strain than the M2e originated from), while naïve mice as well as mice immunized with free fusion protein or PEGylated vectors all succumbed to infection. Non-PEGylated liposomes and free fusion protein induced comparable systemic immune responses, giving rise to higher levels of IFN-γ and serum IgG than the PEGylated liposomes. When investigating the local immune response in the lung, however, it was discovered that the non-PEGylated vector more strongly induced an M2e-specific $CD4^+$ T cell-response as well as higher local secretion of IgA than both the free fusion protein and the PEGylated vector. We further investigated the role of the of M2e-specific $CD4^+$ T cells; following their depletion in mice immunized with the non-PEGylated vector prior to virus challenge, the survival was reduced to 0 %. Thus, the crucial role of cell-mediated immunity for protection was confirmed. Interestingly, while investigating the T cell priming ability, we found that free fusion protein induced strong proliferation of specific $CD4^+$ T cells earlier after immunization than the non-PEGylated vaccine vector. Thus, it appears that the liposome-assisted delivery delays T cell priming but, in the end, leads to a stronger, more effective T cell response. Additionally, we found that the liposomes gave significant IgG titres already after two immunizations, while the free fusion protein required three. This indicates an opportunity for using liposome-assisted delivery to reduce the need for booster immunizations.

In Paper I, we found that immunization with non-PEGylated vaccine vectors better protected from a lethal influenza virus infection than both free fusion protein and the PEGylated vaccine vector. Furthermore, we found that the protective effect correlated with increased activation of the local immune response in the lung. Specifically, we observed slower, but ultimately stronger, proliferation of $CD4^+$ T cells, which proved crucial for the protective effect. Taken together, these results highlight both the importance of inducing local, cell-mediated immunity and the promise of using liposome carriers to improve the immunogenicity of the CTA1-3M2e-DD fusion protein.

7.2 Paper II

In Paper I, cell-mediated immunity proved crucial for the observed protective effect of the non-PEGylated vaccine vector. Priming of $CD4^+$ T cells requires presentation of peptides on cell surface-associated MHC II by DCs (see section 2.2 and 2.3); indeed, the duration and magnitude of such antigen presentation determines the degree and quality of antigen-specific T cell activation.[205, 206] Thus, we chose to focus our investigation in Paper II on how the antigen presentation by DCs is affected by chosen physicochemical properties of vaccine carriers, while simultaneously screening for promising vaccine carrier candidates. In order to observe the kinetics of the antigen presentation, the amount of functionally presented antigen on the DC surface was monitored at various time points after administration of different formulations. The quantification of the antigen presentation was performed using flow cytometry and immunostaining with the YAe antibody, which recognizes the Eα peptide when presented in the MHC II. Therefore, the antigen portion of the CTA1-DD-based fusion protein used in this study consisted of the Eα peptide. With the results from Paper I as a starting point, we attempted to systematically vary physicochemical properties of fusion protein-carrying lipid particles.

Firstly, PEGylated and non-PEGylated POPC-based liposomes were formulated with fusion protein both encapsulated and surface-bound, as in Paper I. During the work presented in Paper I, it proved difficult to tightly control the portion of encapsulated fusion protein. Thus, secondly, the amount of surface-bound protein on non-PEGylated POPC-based liposomes was varied in an attempt to investigate the influence of protein load independently from PEGylation and percentage encapsulated protein. Two different formulations with approximately three times difference in protein load were produced. Lastly, to assess the influence of shape and size, DSPC-based lipodisks, i.e. flat bilayer circles stabilized by their high PEG content,[56] as well as liposomes of a similar composition (abbreviated DSPC-PEG-FP) for direct comparison were produced. The lipodisks were 22 nm in diameter, while the DSPC-PEG were on average 110 nm in hydrodynamic diameter. Out of all the formulations tested, only the DSPC-PEG-FP liposomes, which were in gel phase as opposed to the fluid phase POPC-formulations, led to a substantial increase in presented antigen compared to free fusion protein. For this reason, we decided to more closely investigate the influence of membrane phase state on antigen presentation by focusing on liposome formulations primarily consisting of either gel phase DSPC or fluid phase 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) (Figure

14A). The influence of DOPC-PEG and DSPC-PEG liposomes, both with coupled fusion protein (DOPC-PEG-FP and DSPC-PEG-FP; Figure 14B) and when co-administered with the fusion protein (DOPC-PEG +FP and DSPC-PEG + FP; Figure 14B), on antigen presentation by DCs was investigated. After 24 hours of incubation, DOPC-based liposomes did not significantly improve antigen presentation compared to free fusion protein (Figure 14C). Meanwhile, DSPC-PEG-FP increased the amount of presented antigen by a factor of approximately 8 and DSPC-PEG + FP by a factor of approximately 4 compared to free fusion protein. Interestingly, it was also observed that the increase in peptide presentation induced by the DSPC-based formulations was accompanied by an increase in surface-bound MHC II; however, the YAe fluorescence intensity increase was greater, indicating an improved peptide loading efficiency of DSPC-PEG-FP, and to a smaller extent also DSPC-PEG + FP, compared to the other formulations.

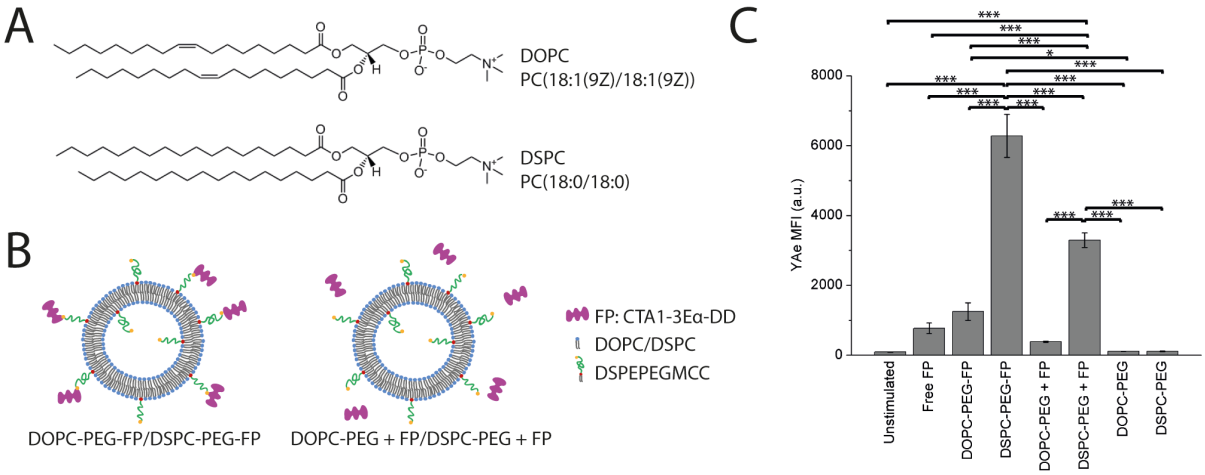

Figure 14. (A) Molecular structures of the main lipids used in the investigation of the influence of membrane phase in Paper II: 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC). These phospholipids are identical with regards to their headgroup and length of their fatty acids, but DOPC has one unsaturation in each acyl chain, causing DOPC-based membranes to be in a fluid phase state while membranes out of the fully saturated DSPC are in a gel phase state at physiological temperature. (B) Schematic representations of the main liposome formulations used: PEGylated DOPC- and DSPC-based liposomes with fusion protein covalently coupled to maleimide at the end of the PEG(2000)-chains (DOPC-PEG-FP and DSPC-PEG-FP), or given alongside the fusion protein (DOPC-PEG + FP and DSPC-PEG + FP). (C) YAe median fluorescence intensity (MFI) of D1 cells after 24 hours incubation with the various vaccine formulations. Error bars show standard error of the mean. Statistical significance: *$p < 0.05$, ***$p < 0.001$.

Additionally, the activation of the DCs, along with their co-stimulatory potential was investigated. Specifically, quantification of the membrane-bound co-stimulatory receptors and activation markers CD80 and CD86 was done by flow cytometry and release of the

relevant cytokines IL-1β (activates T cells, B cells and macrophages[11]) and IL-6 (induces T and B cell activation and proliferation and increases antibody production[11]), was measured by ELISA. Overall, the DSPC-based fusion protein formulations were superior at activating DCs and inducing co-stimulation and cytokine release compared to both DOPC-based liposomes and free fusion protein. Additionally, coupling of the fusion protein to the DSPC-based liposomes proved beneficial for improving cytokine release. Meanwhile, liposomes administered without fusion protein proved poorly immunogenic. Taken together, the results in Paper II showed that DSPC-based liposomes are a promising adjuvant for the CTA-DD fusion protein system, improving antigen processing and presentation as well as co-stimulation, especially when used as a carrier particle.

The observations in Paper II raised questions about what the underlying mechanisms of the efficacy of DSPC-PEG-FP may be. Firstly, increased uptake of this formulation, potentially caused by its lower deformability compared to the others, may be one reason; this possibility was explored in Paper III. Secondly, the ability of DOPC- and DSPC-liposomes to provide opportunity for multivalent attachment, which is important in many processes, including various types of immune recognition, induction of uptake processes, etc. may differ.[207, 208] Thus, a method to explore differences in multivalent attachment, as well as liposome deformation, was developed in Paper IV.

7.3 Paper III

In Paper II, it was suggested that a difference in carrier internalization may have contributed to the observed differences in antigen presentation triggered by DOPC- and DSPC-based vectors. The literature suggests that soft, deformable particles are more likely to get trapped at the cell surface since their envelopment by the cell membrane is energetically more demanding.[135-137] We therefore hypothesized that the DSPC-liposomes were more effective vaccine vectors because they were more readily internalized than their fluid-phase DOPC-based counterparts. Thus, in Paper III, we developed an experimental approach aiming at distinguishing between internalized and surface-bound liposomes. The chosen approach relies on labelling liposomes with the dye DiO. This dye inserts into the lipid membrane with the fluorophore facing outwards (Figure 15A), which in turn allows for quenching of the fluorescence emission with the membrane-impermeable molecule trypan blue (TB). For CLSM imaging as well as to investigate potential effects of the dye on cellular uptake, rhodamine-labelled liposomes, made by inclusion of a small

percentage of rhodamine-labelled lipids when producing the liposomes, were also used (Figure 15A).

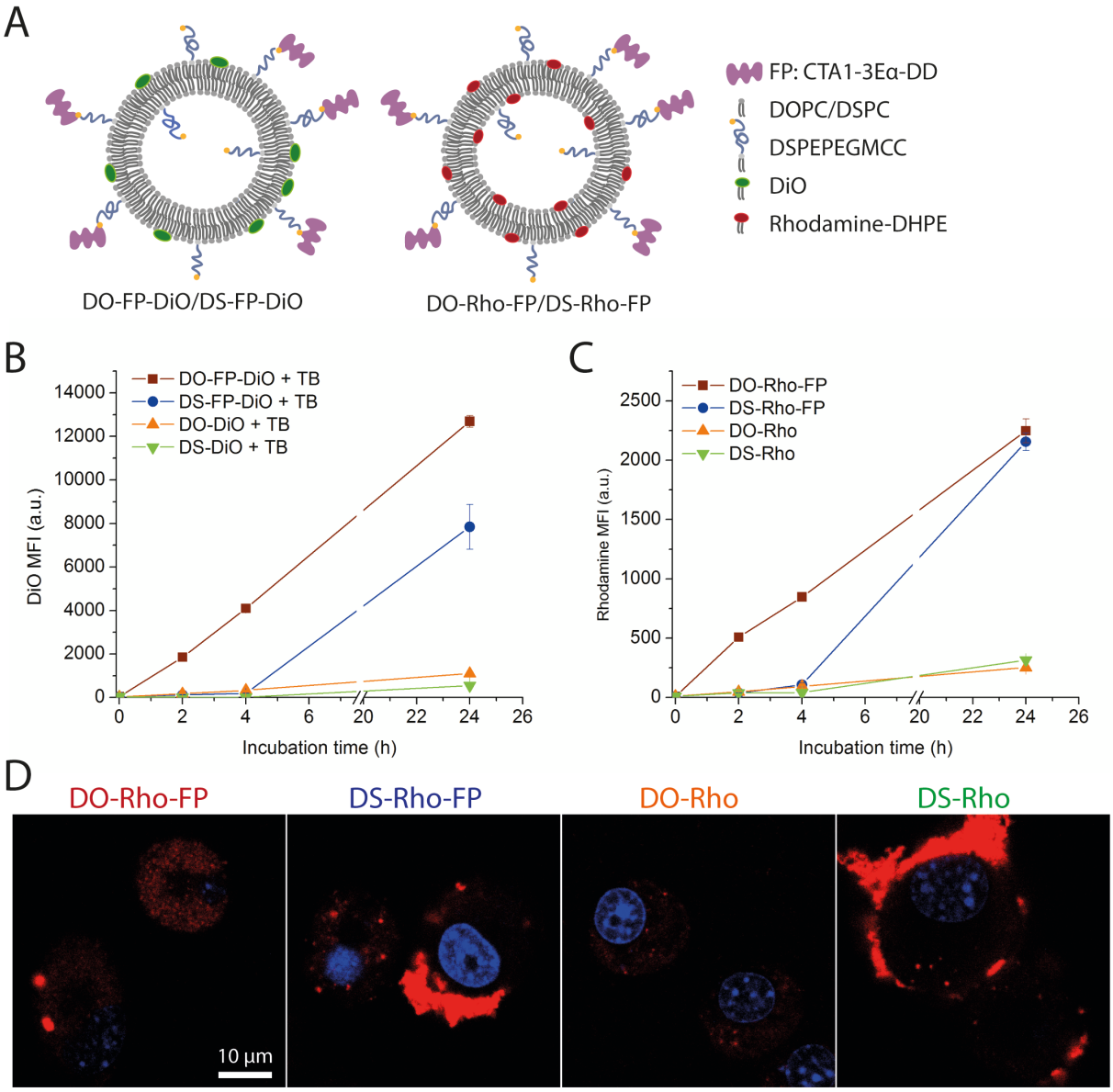

Figure 15. (A) Schematic representation of the fusion protein-carrying liposomes and labelling strategies used in Paper III: post-formation labelling with DiOC18(3) (3,3'-Dioctadecyloxacarbocyanine Perchlorate (DiO) and inclusion of a small percentage of rhodamine-labelled 1,2-Dihexadecanoyl-*sn*-Glycero-3-Phosphoethanolamine (DHPE) lipid. DiO inserts into the lipid membrane with the fluorescent group facing outwards, allowing quenching of extracellularly bound liposomes with trypan blue (TB). (B) Median fluorescence intensity, measured by flow cytometry, of intracellular DiO in JAWSII cells incubated with labelled fluid and gel phase liposomes with (DO-FP-DiO and DS-FP-DiO) and without (DO-DiO and DS-DiO) coupled CTA1-3α-DD fusion protein. Error bars show standard deviation. (C) Median fluorescence intensity of rhodamine associated to JAWSII cells incubated with labelled fluid and gel phase liposomes with coupled fusion protein (DO-Rho-FP and DS-Rho-FP) and without (DO-Rho and DS-Rho), measured by flow cytometry. (D) Confocal laser scanning microscopy (CLSM) images after 24 hours of incubation with the indicated formulations.

When setting out to quantitatively compare fluorescence emission of the two liposome types, we were cautioned by unpublished work by Lubart et al.[67], which specifically observed differences in dye insertion between gel and fluid phase membranes. For this reason, thorough characterization of fluorescence emission was performed and used for normalization of flow cytometry data. In accordance with the findings by Lubart et al., we observed poorer insertion in DSPC-based membranes compared to DOPC-based ones; additionally, and somewhat surprisingly, we observed that the presence of coupled fusion protein on the liposomes had an even greater limiting effect on fluorescence emission. These findings emphasize the importance of mindful use of fluorescent labelling and calls for the further characterization of the liposomes and their labelling. Advanced analytical techniques capable of correlating fluorescence emission with other features, such as size and scattering properties, will be crucial analytical approaches in this regard. We present, in Paper V, a technique capable of doing just that, with single nanoparticle resolution.

To study uptake, cells of the DC cell line JAWSII were incubated with DiO-labelled fluid and gel phase liposomes with and without coupled CTA1-3Eα-DD fusion protein (DO-FP-DiO and DS-FP-DiO; and DO-DiO and DS-DiO, respectively) for various lengths of time up to 24 h, keeping the lipid concentration constant. Flow cytometry analysis revealed steady internalization of the DO-FP-DiO-liposomes, whereas DS-FP-DiO was mainly internalized between 4 and 24 h (Figure 15B). At 24 h, almost the same amount of DS-FP-DiO had been internalized as DO-FP-DiO. Both liposome types, when given without fusion protein, were poorly internalized, indicating that the targeting functionality of the fusion protein was required for effective liposome internalization. Quenching with TB, showed that >80 % of liposomes where internalized at all time points and that there were no noteworthy differences between formulations. This is true with the exception of DS-DiO, which was more poorly internalized in the early timepoints. When instead using rhodamine-labelled liposomes, the uptake behaviour was qualitatively very similar (Figure 15C).

To obtain a more detailed view of the uptake process of rhodamine-labelled liposomes, CLSM imaging was performed. A qualitatively similar kinetic behaviour was observed as with flow cytometry. Additionally, DO-Rho-FP was observed to accumulate on the cell membrane at the early timepoints, followed by efficient internalization. The initial membrane binding was not observed to a significant extent for the other three

formulations. This may indicate that distinct uptake pathways are at play. Furthermore, the DSPC-based liposomes were observed to form aggregates with time, accumulating in cloud-like formations at the outer surface of the cells (Figure 15D). This effect appeared to be dependent on the presence of cells, not just exposure to cell culture medium. Furthermore, the aggregates were removed by the same washing protocol used before flow cytometry analysis, indicating the that the majority of the fluorescence signal in Figure 15C originated intracellularly, as with the DiO-labelled liposomes. Undoubtedly, though, the DSPC-liposome aggregation may have contributed to the distinct kinetics observed for DS-Rho-FP. Interestingly, delayed internalization of gel phase vaccine carriers, correlating with improved immunogenicity of the formulation, has been observed *in vivo* and was attributed to depot formation of the gel phase liposomes.[132] Thus, the aggregation behaviour observed in the DSPC-based liposomes may potentially be beneficial as a means to achieve prolonged release.

It appears that the softer fluid phase liposomes did not, as suggested by literature and initially hypothesized by us, appear to get trapped at the cell surface. Rather, the opposite was observed: DO-FP-DiO and DO-Rho-FP were initially internalized at a faster rate than their DSPC-based counterparts. Thus, although this study was carried out on a different DC cell line than that used in Paper II, the data suggest that a difference in uptake efficiency might not be the main reason behind the differences in antigen presentation observed in Paper II, where DSPC-based vectors increased antigen presentation already before 4 hours of incubation. This, along with differences in how the different liposomes appear to bind to the cell surface, taken together with the differences in peptide loading into MHC II observed in Paper II, indicate that differences in uptake route, and potentially a resulting difference in intracellular fate, may be explanations for our observations. This calls for future investigations of the uptake pathways involved as well as of intracellular trafficking and fate.

7.4 Additional experimental results: Further immunogenicity characterization of DOPC- and DSPC-based vaccine vectors

Intrigued by the results in Paper II and III, we further investigated the immunostimulatory effect of the DOPC- and DSPC-based vaccine vectors. Our intention was to gain insights into whether there was a correlation between the significant increase in activation and antigen presentation in targeted DCs as observed *in vitro* (Paper II) and an enhanced

immune protective effect *in vivo*. For these experiments we used the same immunization protocol as in Paper I; accordingly, mice were intranasally immunized with CTA1-3M2e-DD fusion protein, either free or coupled to DOPC- or DSPC-liposomes, or PBS as a negative control. This was followed by a homo- (X47; Figure 16A-B) or heterosubtypical (PR8; Figure 16C-D) live virus challenge, after which mortality and morbidity were monitored. The results with both virus strains were similar and survival (Figure 16, left panels) and weight loss (Figure 16, right panels) indicated immune protection as compared to the negative control. Most importantly, the liposome-coupled fusion protein provided better protection than free fusion protein, but no difference between the two liposome formulations was observed. The heterosubtypic challenge proved more severe, as expected, with fewer surviving animals and more dramatic weight loss.

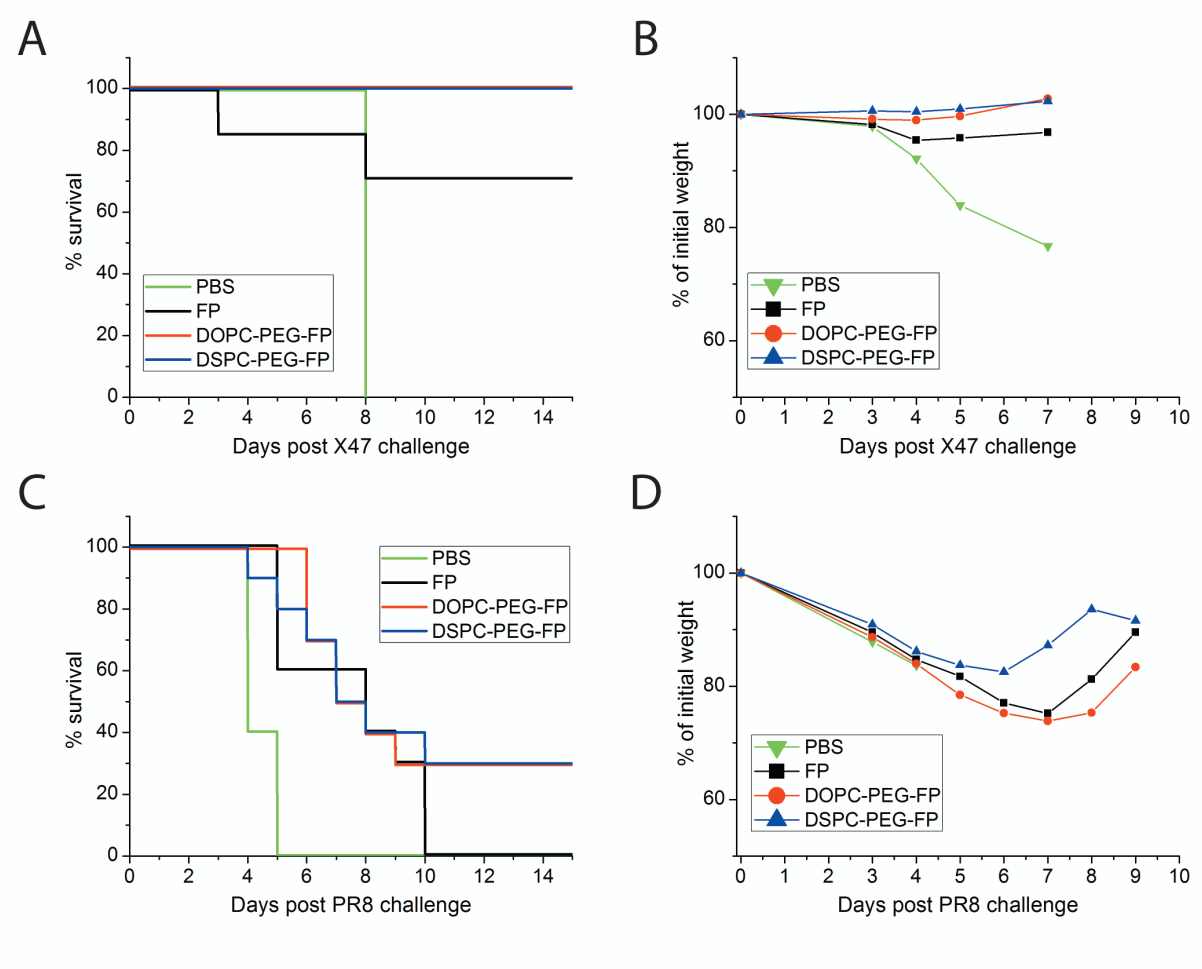

Figure 16. Intranasal immunizations of Balb/c mice were followed by challenge with either the homosubtypic x47 (A-B) or the heterosubtypic PR8 (C-D) virus strain. Survival (left panels) and weight loss (right panels) were monitored over time.

Considering the previously observed difference between DOPC- and DSPC-based carriers *in vitro*, the lack of a differential effect *in vivo* was somewhat unexpected. In an attempt to

shed light on possible explanations for this observation, we examined the release of a wider range of cytokines with ELISA (Figure 17), using the same *in vitro* model system and method as described in Paper II. The cytokines investigated in this manner were IL-22, IL-23, IL-27 and IL-10. IL-22 and IL-23 are greatly involved in maintenance and defence of mucosal barriers.[36, 39] IL-22 was found to be increased by free fusion protein, DOPC-PEG-FP and DSPC-PEG-FP alike, while DSPC-PEG-FP increased IL-23 compared to the unstimulated control. IL-27, which drives regulatory T cells of the Tr1 subtype and has an inhibitory effect on Th1, Th2 and Th17 subset functions mediated by IL-10 production,[40] was most strongly expressed after activation with DSPC-PEG-FP. IL-10 is a major regulatory cytokine,[41] and increased release by DSPC-PEG-FP-activated cells compared to the other formulations could imply reduced protective effector functions. It is not, as such, surprising that vector rigidity would have the capacity to modulate the immune response. Indeed, it has previously been observed that rigid liposomes preferentially induce a Th1-type immune response, while fluid phase liposomes induced a Th2-type response in an *in vivo* leishmaniasis vaccine model.[130] However, the increased release of IL-27 and IL-10, indicating immune-suppressive effects of DSPC-PEG-FP, is surprising considering the promising results in Paper II and that it performs equally well as DOPC-PEG-FP and better than free fusion protein *in vivo* (Figure 16). It should be noted that, since the general trend among all measured cytokines is that they are increased by DSPC-PEG-FP, the net-result may be a reduction of the effector functions due to regulatory cytokines, such as IL-10. Nevertheless, taken together, the additional cytokine data may indicate that altering the physicochemical properties of vaccine vectors may have diverse and difficult-to-predict effects. However, the results with DSPC-based formulations convey optimism as improved immune protection with preserved mucosal barrier functions can be achieved. To what extent mucosal vaccination using DSPC-carriers can have immune-suppressive effects and be used for vaccines against autoimmune diseases warrants further investigations. It does, however, appear that the membrane phase state of liposomes can influence the polarization of the DC response, an aspect which should be further investigated.

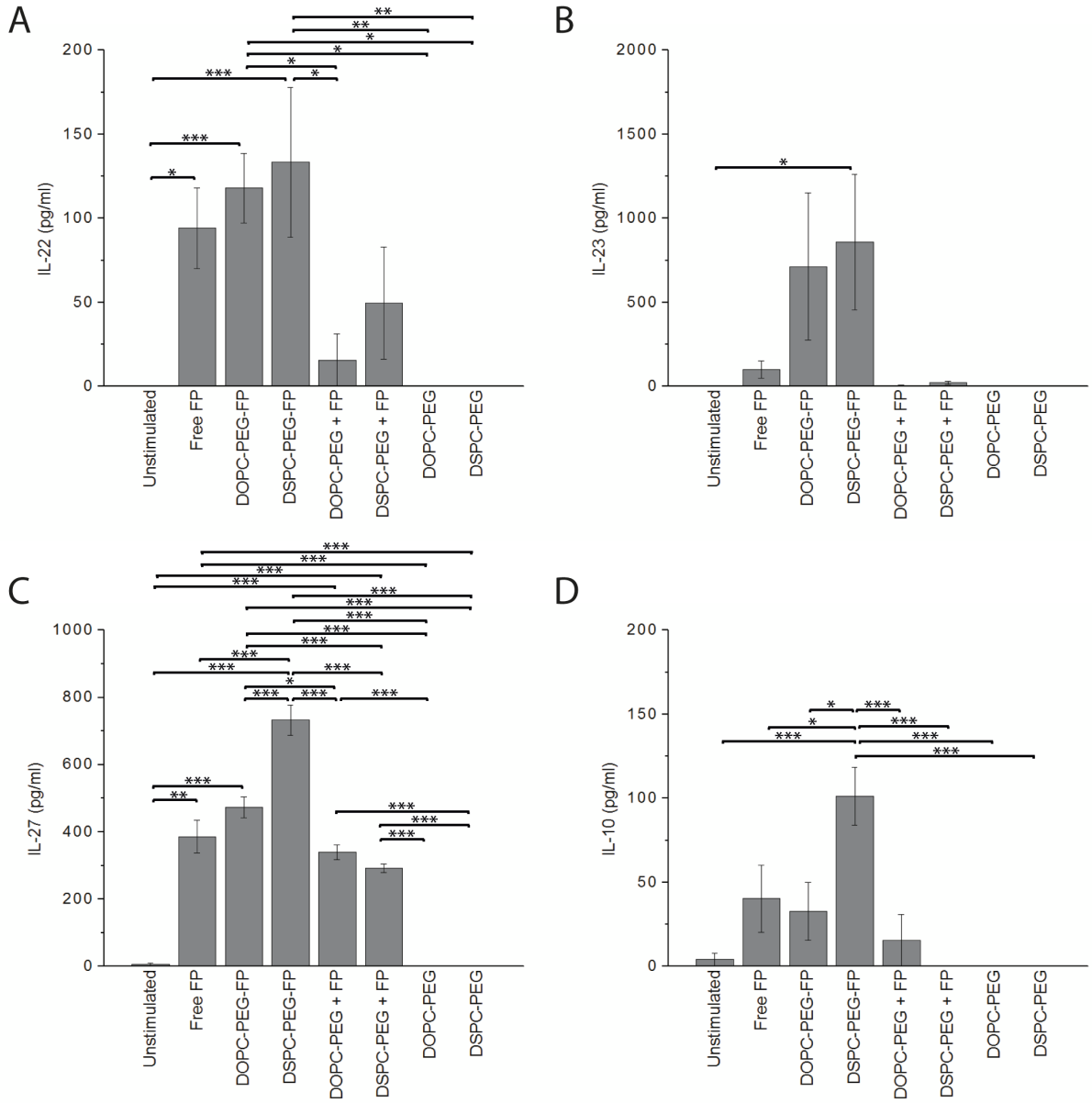

Figure 17. Enzyme-linked immunosorbent assay (ELISA) was used to quantify the amount of (A) IL-22, (B) IL-23, (C) IL-27 and (D) IL-10 in DC cell line culture supernatants following activation for 24 h with PEGylated DOPC- and DSPC-based liposomes with CTA-1-3Eα-DD fusion protein covalently coupled to maleimide at the end of the PEG(2000)-chains (DOPC-PEG-FP and DSPC-PEG-FP), or given alongside the fusion protein (DOPC-PEG + FP and DSPC-PEG + FP), or free fusion protein (FP).

Importantly, DC activation (as investigated in Paper II) is only one element of several that can impact the immunogenicity of an antigen. There are indeed many intermediate steps before the vaccine even reaches the DC and little is known about how, for example, the interaction of liposomes with different kinds of biological fluids, tissues and cells can affect their physicochemical properties. It has been shown that, when using identical liposomes, different administration routes have profound effects on vaccination outcomes,[209] A

possible reason for such effects might be changes to the vector properties depending on the specific microenvironment; an illustrative example of this is the aggregation that DSPC-based liposomes underwent when subjected to biological fluids in Paper III. Thus, such reductionist *in vitro* models as the ones used in Paper II and III are useful for furthering detailed mechanistic understanding but fail to properly emulate the complex, multi-step process of mucosal vaccine delivery and therefore, at this point, to predict *in vivo* efficacy. A move towards using a sequential combination of *in vitro* assays would likely be useful for improving the understanding of the impact of different micro-milieus on particle properties and processes such as cellular uptake, activation, etc. However, such assays need to be accompanied by *in vivo* investigations to understand the relative impact of each element or step on vaccination outcomes to better design future liposome vectors for mucosal vaccination.

7.5 Additional experimental results: TIRF microscopy-based study of cell-particle interactions

As concluded in Paper III, there is likely a difference in how DOPC- and DSPC-based vaccine vectors are internalized. It is, as yet, unknown how the initial interaction between cell surface and vaccine carrier influences initiation and progression of uptake and how that in turn affects the downstream processing of the antigen. For this reason, we aimed to develop a method that allows us to visually, in detail, probe interactions between lipid vaccine particles and cells on a single-particle and single-cell level.

Visualizing movements of single particles requires a high signal-to-noise ratio and high spatial and temporal resolution. To fulfil these requirements, TIRF microscopy was used to visualize the movements of individual particles on the basal cell membrane and to analyse their trajectories. Additionally, we used a topographically patterned substrate with micropillars (Figure 18) to facilitate access to the basal membrane and more uninhibited movement compared to flat glass. As a proof of concept, a mouse-derived foetal skin dendritic cell (FSDC) line[210] was used, as robustness and ease of handling was a priority during the initial development.

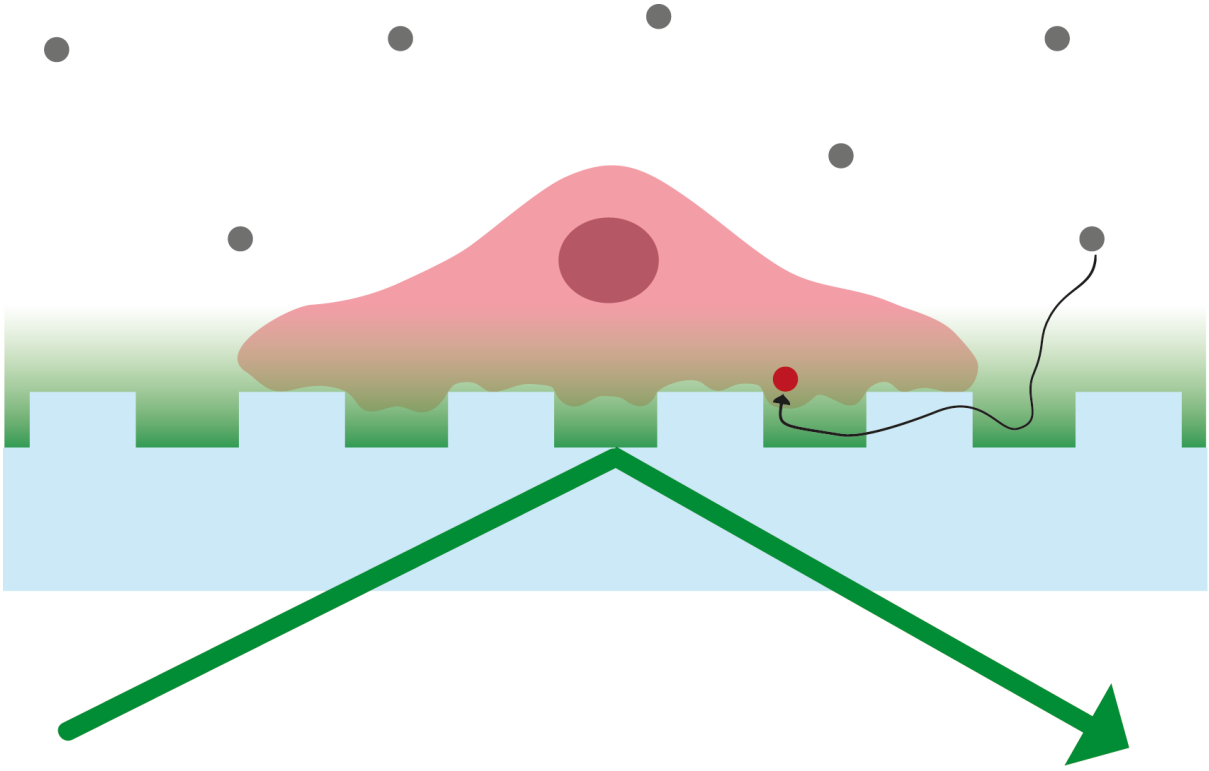

Figure 18. Foetal skin dendritic cells (FSDCs) were cultured on a micropatterned glass substrate with pillars, made as follows: a chromium etching mask was transferred to the substrate by conventional photolithography and lift-off. The pillars were etched in the glass using reactive ion etching. The pillars were 400 nm high and with 1 µm width and spacing, providing relative freedom of movement for lipid particles to interact with the basal cell membrane. The movements of the particles were observed using the surface sensitive technique total internal reflection fluorescence (TIRF) microscopy, which selectively illuminates the volume closest to the glass substrate. Single particle tracking was used to analyse the particle trajectories.

Initially, two different micropatterns were tried: the pillars were 400 nm high and their width and spacing were kept the same at either 1 µm or 2 µm. FSDCs were cultured on flat glass and the two kinds of topographically patterned substrates. The cells were fixed, permeabilized and stained with DAPI and rhodamine phalloidin (labelling nucleus and F-actin, a cytoskeleton marker, respectively). The morphology of the cells was inspected using epifluorescence microscopy. Since these cells are normally cultured on flat substrates, the flat glass sample was considered the positive control (**Figure 19**A). Here, cells were seen to stretch out, forming thin filaments to outstretched attachment points. A similar morphology was seen on the 1µm pattern (**Figure 19**B), while on the 2 µm pattern cells appeared more rounded and with fewer outreaching filaments (**Figure 19**C). Since the cells exhibited normal morphology on the 1 µm pattern, this was chosen for use in the assay.

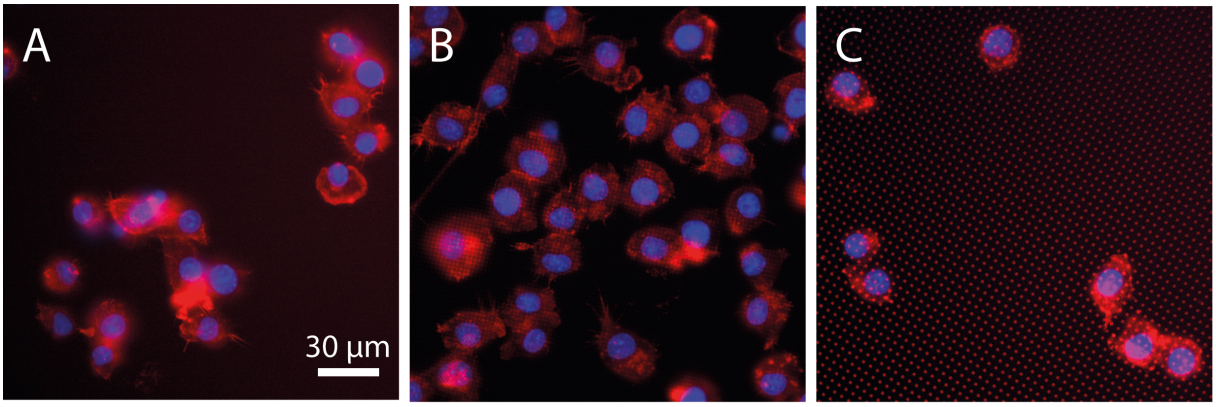

Figure 19. Foetal skin dendritic cells (FSDCs) stained with DAPI (nucleus, blue) and rhodamine phalloidin (F-actin, red), cultured on: (A) flat glass, or glass with 400 nm high pillars with either (B) 1 µm width and spacing or (C) 2 µm diameter and spacing.

In order to study the interaction between lipid particles and cells, FSDCs were incubated with rhodamine-labelled fusion protein-functionalized liposomes (**Figure 20**A). The cell membrane was labelled with PKH67 in order to visualize the cells (**Figure 20**B). The imaging was performed at the level of the top of the pillars, by focusing on the thin layer of chromium deposited there in the manufacturing process. The field of view is the same in **Figure 20**A and B; thus, the movement of the individual particles tracked using single particle tracking in **Figure 20**C is taking place on the cell surface. Time-lapses were recorded at a speed of 5 frames per second. No particles showed the random movement patterns associated with free diffusion. Over short time scales, particles exhibited confined movement (see inset in **Figure 20**C). Over time scales of several minutes, directed movement patterns could be observed (see main image in **Figure 20**C). It was however difficult to determine if these were decoupled from movement of the cell itself. Particles were occasionally observed to flicker in and out of the focal volume, which could be explained by the fact that the normal morphology of DCs is not smooth: they generally have a multitude of protrusions (dendrites) that range from being filamentous to being more skirt-like depending on the level of activation.[211] Thus, it can be expected for particles to exhibit considerable movement also in the z-direction.

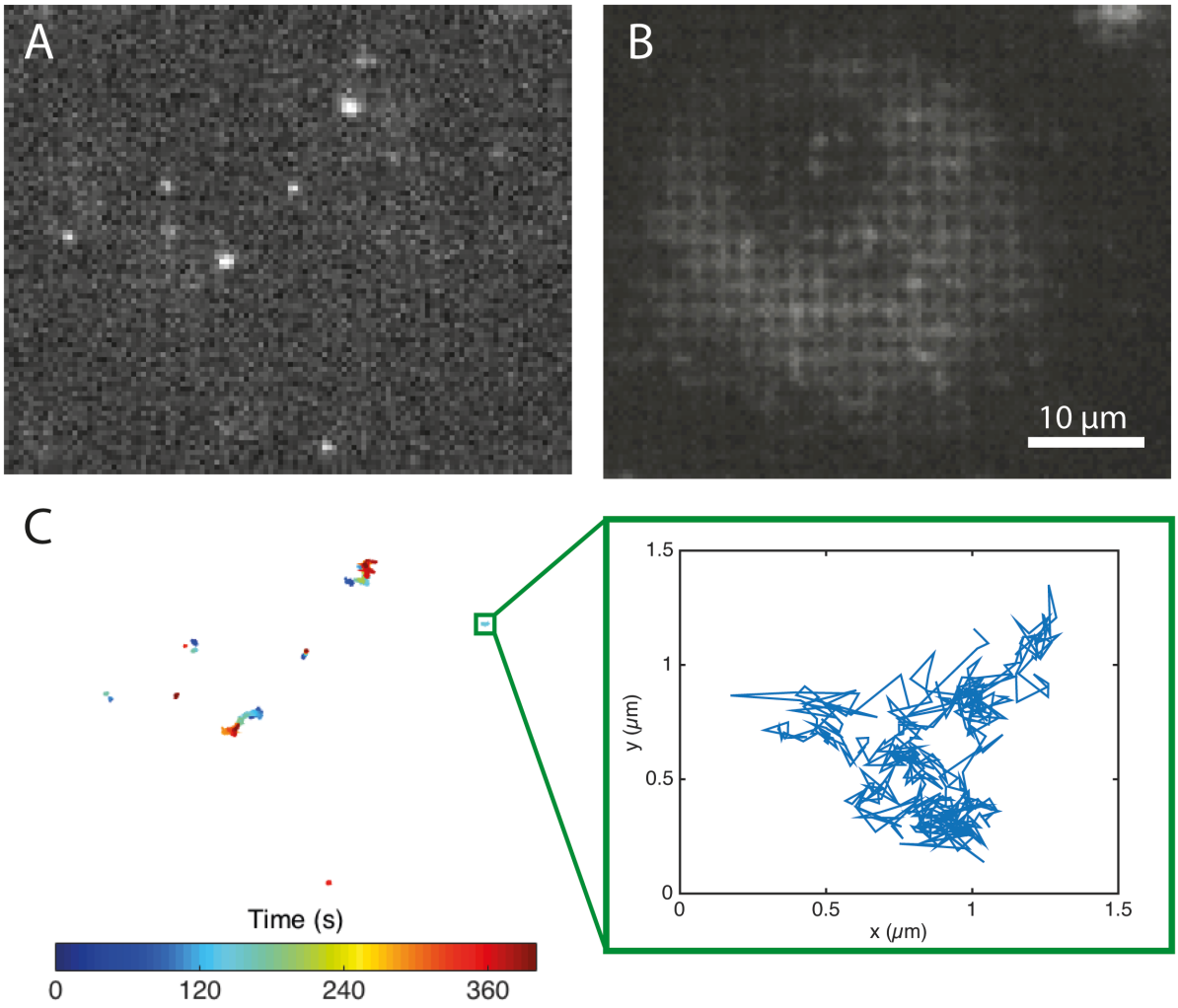

Figure 20. (A) Total internal reflection fluorescence (TIRF) micrograph of rhodamine-labelled fusion protein-functionalized liposomes (same formulation as the non-PEGylated vectors in Paper I) attached to the surface of the PKH67-labelled foetal skin dendritic cell (FSDC) seen in (B). (C) Trajectories over time of the liposomes in (A). Inset shows detailed track. The particles were tracked using a dedicated MATLAB script created within the division of Biological Physics, Chalmers University of Technology (available upon request).

In conclusion, we have developed a tool that enables study of the interaction between vaccine carriers and the cell surface, which is the first step in the uptake process. From the particle trajectories, it is possible to calculate diffusion rates, which could give indications about differences in the valency of the attachment. Due to the dynamic and complex nature of the cell membrane, simplified models are needed to obtain detailed understanding of the impact of valency on particle binding. For this reason, in Paper IV, we developed a method that enables the study of binding valency and particle deformation using SPR and well-defined cell membrane mimics. Combining the type of information obtainable with these

assays, we could characterize how changes to the particle properties influence the observed membrane interactions in a quantitative fashion. Correlation of such information with observed differences in uptake (Paper III) and additional characterization of uptake pathways and intracellular fate could help elucidate the mechanistic impact that changing particle physicochemical properties has on different stages of interaction with DCs.

7.6 Paper IV

In the literature, there are contradictory results about the influence of membrane rigidity (gel or fluid phase) on immunogenicity[108, 125-134] and cellular uptake.[212, 213] Theoretical[135-137] and experimental[131, 137] investigations suggest that high particle rigidity should be used to optimize cellular uptake; simultaneously, high ligand density has also been shown to enhance cellular internalization and impact the cellular uptake pathway.[214, 215] In liposomes, these features may be at odds with each other: gel phase liposomes are more rigid than fluid phase ones, while a membrane in the fluid phase state acts as a 2D liquid, with inherent movement of bound ligands, which may allow for locally increased ligand density. It is thus hard to predict the relative importance of rigidity and fluidity for liposome-cell membrane interactions and these properties are, additionally, not trivial to quantify. In an attempt to address this question, we show in Paper IV an SPR-based method developed for quantifying surface-induced deformation and binding multivalency, and used it to study liposomes of different membrane phase state.

As supported cell membrane mimic, we utilized a silica-supported lipid bilayer with varying amounts of cap biotin, to which streptavidin was bound as a model receptor. Gel (DSPC) or fluid (DOPC) phase liposomes with a constant ligand (PEG-biotin) density and equal size in suspension were subsequently introduced, and the bound mass and film thickness of the various layers were simultaneously quantified with multiparametric SPR (**Figure 21**). Although simplistic, the well-defined nature of this model system allowed for precise quantification of the number of bound entities (streptavidin molecules and DSPC- or DOPC-based liposomes) and thus the number of receptor-ligands pairs involved in the liposome binding. By varying the amounts of cap biotin in the supported lipid bilayer, low and high streptavidin coverages were achieved and liposome binding was investigated under conditions where the number of available receptors in the cell membrane mimic were or were not a limiting factor.

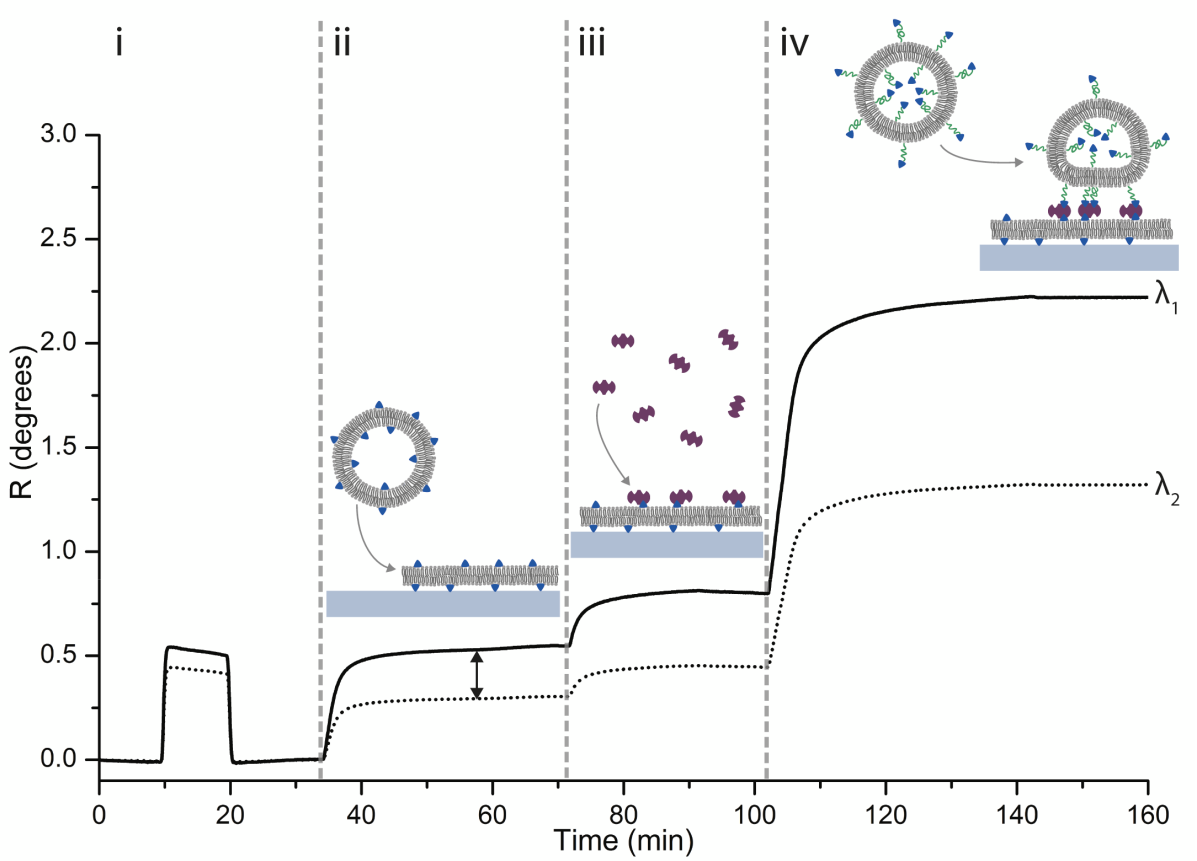

Figure 21. A typical dual-wavelength surface plasmon resonance (SPR) measurement with schematics of the different steps: (i) injection of 5 wt% glycerol for calibration purposes, (ii) injection of liposomes and formation of a supported lipid bilayer with cap biotin, (iii) binding of streptavidin and (iv) binding and deformation of DOPC-PEG-biotin or DSPC-PEG-biotin liposomes.

At low streptavidin coverage, where all available streptavidin molecules were recruited in liposome binding, each individual fluid and gel phase liposome interacted using approximately 80 and 50 streptavidin-biotin pairs, respectively. This was enough to cause a decrease in the height of the fluid phase DOPC-PEG-biotin liposomes by approximately 20 % compared to the gel phase DSPC-PEG-biotin, which appeared to remain undeformed. At high streptavidin coverage, the liposome binding was not limited by the number of available receptors, but rather by geometrical constraints. In this case, it is not possible to precisely know the number of engaged receptor-ligand pairs per liposome, but it could be approximated, using the measured streptavidin coverage and liposome film thickness, and geometrical considerations (deformation to a truncated sphere shape), that at least 110 and 30 streptavidin molecules would fit under the footprint of the fluid and gel phase liposomes, respectively. Further, in this regime, DSPC-PEG-biotin deformed less than 10 %, while DOPC-PEG-biotin reduced more than 30 % in height. Considering the number of available ligands on each liposome (approximately 240 outwards-facing) and

that each streptavidin molecule would reasonably be able to accommodate two PEG-biotin ligands, it is likely that almost all of the available ligands on DOPC-PEG-biotin were engaged in binding at high streptavidin coverage. For DSPC-PEG-biotin, which utilized a much smaller portion of its available ligands in binding, the low mobility of the ligands, i.e. the low fluidity of the membrane, appeared to be the limiting factor for deformation.

In summary, we used a well-defined model of liposome-based vaccines and quantified binding and deformation upon interaction with a supported cell membrane-mimic using multiparametric SPR. Using two different receptor coverages, we were able to quantify surface-induced deformation of liposomes in different phase states, and also demonstrated that the method can be used to decouple effects related to membrane fluidity and rigidity. We are well aware that in the context of vaccine carriers, the streptavidin-biotin model is very simplistic; however, an advantage of the supported lipid bilayer-platform is that it can easily be customized, and can be transferred to more complex membrane mimics. For example, cell membrane-derived supported lipid bilayers[216] could be introduced, allowing for studies of liposome deformation in much more biologically relevant scenarios. Thus, the method presented in Paper IV and the TIRF-based assay introduced in section 7.5 could serve as excellent complements to each other in the work towards understanding the initial interaction between liposomes and the cell membrane.

7.7 Paper V

Paper V focuses on the characterization of extracellular vesicles (EVs) and I contributed to this work by guiding the use of self-inserting fluorescent dyes (similar to those used in Paper III) to modify EVs for fluorescence microscopy visualization. EVs are a type of natural delivery vehicle: a lipid vesicle interspersed with membrane proteins, often carrying both nucleic acids and soluble proteins in the aqueous core.[217] They are released by most cell types and play an important role in intercellular signalling; in recent years, interest in their potential as a drug and vaccine delivery platform has surged.[217-221] However, due to their biological origin, one has little control over their composition and physicochemical properties. Thus, to be able to fully utilize their potential, or indeed be able to produce synthetic substitutes, classification of different subpopulations is critically needed. The vast complexity in EV origin, biological function, biomolecular composition and physical structure emphasizes the importance of methods capable of single-particle characterization with single-nanoparticle resolution. To this end, we utilized surface-sensitive waveguide

scattering microscopy, a technique with single vesicle resolution, capable of simultaneous scattering and fluorescence detection. The technique is schematically represented in Figure 22A, and was used for characterization of the correlation between the fluorescence emission originating from insertion of the lipophilic dye PKH26 and the (label-free) scattering emission of EVs, a parameter that is strongly dependent on EV size (volume). The EVs in question, originating from human mast cells, form two distinct subpopulations when separated based on buoyant density using a gradient of different concentrations of iodixanol and sucrose. The aim was to ascertain whether there were differences in the optical properties, especially dye insertion efficiency and the refractive index, of the high-density (~1.27 g/cm^3) and low-density (~1.15 g/cm^3) populations. Given that the two populations were of comparable size (109 and 97 nm modal diameter for the high- and low-density sample, respectively), higher density was expected to correlate with higher effective refractive index and, thus, higher scattering intensity. However, the high-density population proved to display a factor 3 *lower* modal scattering intensity (Figure 22B, right histogram) compared to the low-density population. Further, the positive correlation between fluorescence and scattering intensities within each EV sample suggested an increase of incorporated dye molecules with increasing vesicle size, as expected (Figure 22B, main panel). However, the fluorescence intensities differed between the two EV samples, with the high-density sample exhibiting lower fluorescence intensity, despite their somewhat larger modal size. This might be due to a higher protein-to-lipid ratio in the high-density sample; however, this is unlikely considering that it had lower effective refractive index, while proteins have a higher refractive index than lipids. Alternatively, the divergence in fluorescence intensity could be due to a difference in labelling efficiency, highlighting the importance of understanding the influence that particle properties may have on labelling efficiency, as also recently described by Lubart et al.(in review)[67], as well as in Paper III. Indeed, the waveguide microscopy technique may be useful to gain further understanding, on a single-particle level, of the physicochemical mechanisms governing membrane-insertion of lipophilic dyes.

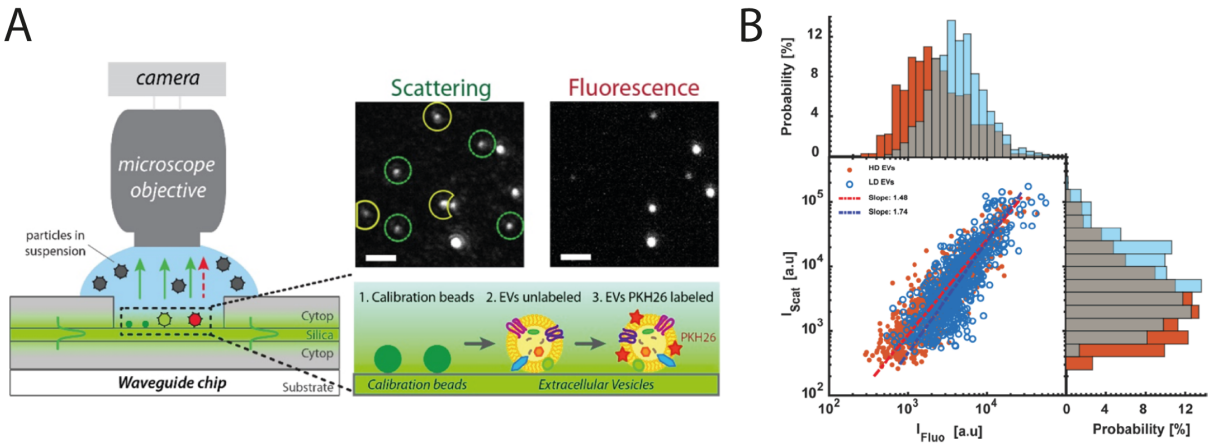

Figure 22. (A) Schematic illustration of the waveguide microscopy setup, utilizing a waveguide chip consisting of a silica core encapsulated by a CYTOP cladding layer on both sides, illuminated by the means of linearly polarized laser light coupled via an optical fiber.[222] Partial removal of the top cladding layer creates a silica sensing surface on which a sample droplet is placed and imaged via a water immersion objective. CYTOP and water are closely matched with regards to refractive index (1.34 vs 1.33, respectively), allowing for undisrupted propagation of the evanescent wave along the solid-liquid interface.[223] The resulting exponentially decaying illumination profile allows for visualization of surface-adsorbed particles with high signal-to-noise ratio simultaneously in both scattering and fluorescence mode. (B) Top: PKH26 fluorescence intensity distribution of high-density (HD) and low-density (LD) EV populations. Right: Scattering intensity distribution of HD and LD EV populations. Main: Correlation between the scattering and fluorescence intensities for EVs simultaneously visualized in scattering and fluorescence mode (log-log plot).

In summary, Paper V highlights the importance of characterization and classification of EVs, and illustrates the benefits of novel techniques, such as the one used therein, for characterization of properties that are not commonly reported. Specifically, it can in the future help guide the use of self-inserting dyes of the type that was used in Paper III and might assist in the further understanding of such results as are presented there.

8
Conclusions and outlook

In this thesis, I set out to further the understanding of how physicochemical properties of liposomal vaccine carriers influence the development of protective immunity. When working towards this purpose, we identified specific needs for characterization of physicochemical properties of the liposomes and explored varying liposome formulations in attempts to improve the immunogenicity of fusion proteins based on the mucosal adjuvant CTA1-DD. Although we reached some insights during this work, many new questions were also raised. In this chapter, some of the questions and opportunities to be addressed in the future are presented. The chapter is divided into three parts where, firstly, future needs for further characterization of nanoparticle properties are identified. Secondly, opportunities for further development of liposome-based vaccine formulations are suggested and, lastly, future approaches to vaccine research are discussed.

8.1 Future opportunities for physicochemical characterization of liposomes

The general focus of this thesis has been on trying to understand the connection between physicochemical properties and liposome immunogenicity; most efforts have been devoted to systematic investigations aimed at understanding the influence of membrane phase state. This is a complex task as the interplay between liposome rigidity and fluidity is not fully understood, which likely contributes to the conflicting results both in our work and in the literature (see section 5.5). In this respect, it appears that both particle rigidity, a characteristic feature of gel-phase membranes, and high ligand density in the particle-cell contact, which can be facilitated by high fluidity (as shown in Paper IV), could be beneficial. This may call for specific decoupling of the effects of these two properties in order to improve efficacy of vaccine vectors. This is not a facile undertaking, though, as it would require, for example, the encapsulation of a rigid core in a lipid bilayer with maintained fluidity, but is, on the other hand, likely to yield valuable insights that are currently lacking.

Another aspect that that could influence the binding avidity and vector deformability is related to surface functionalization, antigen coupling in general and the use of flexible linkers for protein coupling in particular. This is a point of particular interest, considering that the formulations used in Paper I differed mainly with regards to PEGylation and number of coupled fusion proteins. Both of these properties could most likely impact the way that the liposomes bind to and deform on the cell membrane, although it is difficult to predict exactly how. Investigations of such questions place high demands on the understanding of how particle composition and functionalization impact the dynamics of nanoparticle structure upon interaction with cells. For this, novel analytical techniques and model systems for particle characterization are required, and the platform presented in Paper IV represents an interesting opportunity to further the understanding of the relations between nanoparticle composition, structure and function, especially since the method should be compatible with the TIRF-based cellular imaging presented in section 7.5.

In attempting of further the understanding of the connection between physicochemical properties of particles and their biological function, we have made use of simplified systems for the specific study of individual events or interactions within the immune response. An approach common to all our studies, as well as in most others, relies on carrying out an initial characterization of the vaccine vectors and assume that their properties remain unchanged throughout the biological experiment subsequently performed. It is becoming increasingly clear, though, that such an assumption does not necessarily hold. This is illustrated, for example, in Paper III, where DSPC-based liposomes were observed to form large connected structures upon exposure to cells. Furthermore, it is becoming increasingly clear that a protein corona is formed upon nanoparticle exposure to biological fluids.[224] This aspect must be considered in future attempts at rationalizing the design of nanoparticle-based vaccine formulations and may, in fact, be intentionally taken advantage of.[224] As a matter of fact, protein corona-formation could explain the fact that liposomes that are seemingly innocuous on their own, appear to have immunostimulatory properties even when they are not intentionally used as antigen carriers. For example, liposomes have previously been observed *in vivo* to have an adjuvant effect even when given up to 48 h before the antigen.[117] In the context of our research, it is worthwhile to note that, in Paper II, DSPC-based liposomes, which are non-immunogenic when administered alone, improved antigen presentation and DC activation compared to fusion protein alone, when co-administered with the protein. This may suggest that fusion protein might have

spontaneously adsorbed to the liposomes independently of intentional covalent coupling. These observed and hypothesized changes to the original physicochemical properties of liposome formulations upon contact with various biological model systems, emphasize the importance of considering and investigating, not only the initial physicochemical profile of vaccine vectors, but perhaps even more importantly, how they are changed by interaction with various micro-milieus. In the future, investigations into the nature and content of the protein corona, as well as potential differences in how coupled protein may solubilize from liposome formulations of interest when subjected to complex media, should be conducted.

8.2 Future directions in lipid-based vaccine vector design

During the work described in this thesis, we found that the non-PEGylated POPC-based vaccine vectors perform better than their PEGylated counterparts *in vivo* (Paper I) and that gel phase DSPC-based vectors were superior to fluid phase ones in the *in vitro* DC model system used in Paper II. Although much is left to understand about the mechanisms underlying any of the improved functions that we have observed, I here present some suggestions for possible future directions in vaccine vector design, with a basis in our findings and in the reports of others.

Targeting of vaccine formulations to certain cell types, such as DCs, is a core concept in many attempts to create successful mucosal vaccine formulations (see section 5.7), including ours. The DD moiety is included in the fusion protein to provide DC targeting abilities, and indeed, in Paper III, it was shown that fusion protein was necessary for efficient cellular uptake of liposomes. However, it is not known how the DD-portion of the fusion proteins employed in this work targets DCs. For this reason, the efficiency and specificity of the targeting can likely be improved by replacing the DD-portion with a ligand targeting a known receptor. Indeed, the targeting moiety could be chosen to target a specific DC subset (see section 2.2) depending on the type of response desired.[225] Changes to the fusion protein aside, the advantage of using a liposomal carrier is that other components can be incorporated, in addition to vaccine antigens. Lewis X oligosaccharides (sugar polymers) have been used to target DCs and as adjuvants[226] and it might be interesting to incorporate a lipid-anchored variant in liposome-based vaccine formulations. Similarly, TLR-ligands have been used to target DCs (see section 5.7) and it has been shown that using a combination of ligands for both cell surface- and endosomal

TLRs induce markedly more effective responses against viral challenge by increasing the quality, rather than the quantity, of the Tc cell response.[227] Liposomal carriers are excellent candidates for combination with such approaches to modulation of the immune response, as they provide opportunity for biomimicry; one may display ligands for cell surface TLRs on the exterior of the liposome, while packaging endosomal TLR ligands inside. Thus, it would be interesting to use liposome-associated ligands, not only for cell targeting, but also for intentional modulation of the immune response in the future development of vaccine carrier formulations.

8.3 Future approaches to vaccine research

We have, for the most part, taken a reductionist approach to the investigations presented in this thesis. The usefulness of such work for furthering the mechanistic understanding of biological events as well as the importance of careful characterization of vaccine vectors has, I believe, been illustrated. However, while we collect the pieces of the "how physicochemical properties of vaccine vectors influence the development of protective immunity"-puzzle one by one, it is not trivial to assemble them into a whole, coherent picture. Especially in the case of mucosal administration there are many intermediate steps (contact with biological fluids, passage through mucosa, intracellular transport, to name a few (see chapter 2)) between administration and measurable outcome, with incomplete understanding of how earlier steps may influence latter ones, and almost no knowledge exists with respect to how they may influence the physicochemical properties of the particles. Thus, I believe efforts should be made to generalize and contextualize the mechanistic understanding, as has been done in related fields. For example, Miao et al. utilized combinatorial lipid libraries to identify promising mRNA delivery vehicles and to identify shared common structures among promising lipids.[228] Similarly, Wong et al. assembled a library of oil-in-water nanoemulsion-based mucosal adjuvants, which were evaluated using a series of high-throughput *in vitro* assays, attempting to establish connections between physicochemical properties, key biological events, and *in vivo* immune responses.[229] Additionally, in later years, systems vaccinology has emerged as a promising new field, where systems biology is applied to the challenge of designing and evaluating new vaccine candidates.[230] Thus, I believe that, in order for great strides to be made in the area of study outlined in this thesis, further mechanistic understanding should be combined with, or complemented by, systematic, large-scale screening studies and/or systems biology approaches.

9
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